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VARIATION AND FILTRABILITY STUDIES ON B. MESENTERICUS AND B. VULGATUS[1]

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INTRODUCTION

Bacterial variation has, since the dawn of modern bacteriology, been a subject of more than passing interest, and within the last decade it has ventured to occupy the front stage. The recent awakening of interest was stimulated by new approaches in this field of research, and by vigorously renewed claims regarding the occurrence of so-called "life cycles" and "filtrable phases" of bacteria.

The present investigation was suggested partly by the work of Smeaton and Rettger (1924) in which a strain of *B. mesentericus* isolated from a case of clinical pulmonary tuberculosis was observed to undergo extreme pleomorphism and variability in staining properties, under certain conditions of cultivation and incubation. It was stimulated further by the writers' observation of marked colonial variation in a laboratory stock strain of *B. mesentericus*.

Very few references to papers dealing with variation of *B. mesentericus* and *B. vulgatus* are found in the literature. Two publications, one by Seligmann (1919) and the other by Kerckhoff (1927) describe mucoid forms of *B. mesentericus*. Gee (1927) observed smooth and rough forms of an aerobic spore-producing organism resembling *B. vulgatus*, and Wahlin (1930) reported a dissociation study of a vulgatus-like bacillus. A few attempts have also been made to demonstrate filtrability of members of the subtilis group. The most recent were those of Rettger and

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[1] This paper covers in part the work done by the senior author as partial requirement for the degree of Doctor of Philosophy in Yale University.

Gillespie (1933), who failed to demonstrate filter-passing properties. Haag (1927), claims to have observed a filtrable phase or form which was derived from a culture of *B. anthracis*.

The main interest in the present investigation lay in attempts to demonstrate filtrable phases or stages of *B. mesentericus* and *B. vulgatus*. For a more extensive review the reader is referred to the doctorate thesis of the senior author, deposited in the Yale University Library.

SOURCES OF STRAINS USED, AND COLONIAL TYPES EXHIBITED AT THE OUTSET ON BEEF-INFUSION AGAR AFTER FORTY-EIGHT HOURS INCUBATION AT 30°C.

B. mesentericus-F. Obtained originally from Ford, and carried for several years as a laboratory stock culture. This was split into the four major variants more easily than were any of the other original strains, and the variants were more easily stabilized. Colonies of the rS colonies yielded only the rS type. The smooth intermediate was regarded as the so-called "normal" type.

B. mesentericus-Am. M. Obtained originally from American Type Culture Collection, and carried in stock for about ten years. It showed more variation at the beginning than any of the other strains. A great majority of the colonies were of the rS type. This type was readily preserved by direct transfer to new agar.

B. mesentericus-4519. From American Type Culture Collection. About 95 per cent of the agar colonies were of the rS type, at the outset.

B. mesentericus-M. From H. J. Conn. Obtained originally from Dr. Meyer, Marburg. It produced only the rS type of colony, but differed from the previous rS strains in that the colonies were more dry and were not so easily removed from the agar plates. Strain M was the most stable of all of the organisms studied.

B. mesentericus-JH4 and *B. mesentericus*-JH4A. From Ford. These proved to resemble very closely the F strain. The rS type was readily established as stable.

B. vulgatus-123–4529. From American Type Culture Collection. It produced two types of colonies, rS and Rs. The rS greatly predominated, and was regarded as the "normal" type for this strain. It resembled very closely the rS form of the Marburg strain (M).

B. vulgatus-JH3 and *B. vulgatus*-JH3A. From Ford. The rS type of colonies of these two strains greatly predominated. They were easily established as stable or permanent.

All of the nine strains, when grown on the routine agar medium at 30°, and when stained by the Gram method, showed characteristic Gram-positive rods. Most of the cells were single; occasionally there were short chains. The cells were moderately motile. Spores were observed after twenty-four hours incubation at 30°C.; many were present at the end of forty-eight hours. The spores were oval and equatorial, and did not cause bulging of the sporangium, except rarely. Frequently, small portions of the sporangium were seen on the free spores.

MEDIA AND METHODS

The media used for most of the routine procedures were standard beef-infusion broth containing 1 per cent bacto-peptone and 0.5 per cent NaCl, and an agar medium of the same composition plus 1.8 per cent agar. The final reaction was pH 7.5. Other media employed were prepared according to standard methods.

PRODUCTION OF VARIANTS

Various attempts to derive comparatively stable variants from the "normal," rS, type by continued incubation in deep infusion broth failed; nor did the addition of 1 per cent normal rabbit's serum help to bring about definite transformation of type. Some variation was observed when platings were made on nutrient agar, but the organisms tended quickly to revert to the rS type. Rapid and frequent transfer to infusion broth also yielded practically negative results.

By far the most satisfactory method of producing and stabilizing the variants was the selective isolation of colonies from agar plates. Until a desired colony type had been obtained and purified, re-isolations were made every twenty-four hours. After a new type was once stabilized, transfers were made only every four or five days. The method was as follows: the "normal" stock (rS) strains were streaked on infusion agar plates and, after incubation for twenty-four hours at 30°C., colonies were selected which were either "smoother" or "rougher" than the "normal." These were streaked on fresh plates. Characteristic rS colonies were carried forward at the same time. After the plates had been incubated for twenty-four hours the same procedure of selection and transfer was repeated. In this way distinct colony types were well separated from one another after many subcultures. At first these types were not thoroughly stable. When they were not transferred at the end of twenty-four hours they frequently reverted to their original form. On the other hand, after numerous selective transfers, the derived types showed marked stability.

Following this method, the four major colony types (S, rS, Rs and R) were developed without much difficulty. The Rs type was obtained most easily of all. A stabilized Rs form was produced within three or four transfers from all of the nine original strains. The extreme smooth or S type was the most difficult of all to obtain. At least ten or twelve transfers were required for the production of an even moderately stable S colony. In one culture (Marburg) a smooth type was never stabilized, although it was occasionally observed. The R colonies were very easily developed, but the most careful selection was necessary to maintain their extreme roughness throughout the series of subsequent transfers. The different variant colonies were carried through 150 culture generations.

No great effort was directed toward producing phantom or mucoid forms. Mucoid colonies were very frequently observed, however. These, except for their mucoid character, were quite similar to the smooth intermediates in appearance. Phantom types were also found quite often. They were suggestive of the types described by Soule for *B. subtilis* (1928), and by Nungester (1929) for *B. anthracis*, except that they reverted to the normal type more readily than theirs.

Description of four major colonial variants

The S colony of these species has an even compact margin, is free from surface markings, and is elevated above the surface. It has a diameter of about 2 mm., is grayish white in color and of a rather soft or butyrous consistency. With the exception of strain JH3, which was dry and smooth, the colonies are quite moist. They are opaque throughout and glisten more or less when examined under a bright light. The S type was obtained from all of the nine parent strains, and was stabilized in all except the "Marburg" *B. mesentericus* strain (see fig. 1).

The rS colony is quite similar to the S type in several respects. It is opaque, glistens in a strong light, is raised and is grayish-white in color. Its margin is entire, but is characterized by small indentations which give it a rather wavy appearance. The surface of the colony is marked by fine convolutions (see fig. 2).

The Rs colony is noticeably flatter than either of the two types just described; it is dull and somewhat grayer in color; it shows, on examination with a hand lens, the "ground-glass" appearance frequently described for members of the group. Its margins are extensively indented, and its surface is lined and furrowed. Occasional small groups of cells are seen along the margin of the colony, producing an irregular, ragged appearance. Colonies of this type are, as a rule, somewhat larger than the smooth or S type (see fig. 3).

The R colony is very much like the Rs, but more exaggerated in every detail; it is flatter, more spreading and duller than the Rs colony. Examination with the low power objective shows a very uneven, irregular margin which closely approximates in appearance the "Medusa-head" growth described classically for *B. anthracis*. The whole surface is furrowed and lined, as are those of the Rs colonies. With a hand lens the "ground-glass" character is seen to be particularly marked (see fig. 4).

Stability of variants

A brief study was made to determine the degree of stability which had been developed in the variants. The different types were inoculated into several different media, incubated, and examined at various intervals. The media included plain nutrient broth, glucose and mannitol broth, broth containing 2 per cent normal rabbit serum, and broth tubes of different H-ion concentrations (pH 5.0, 6.0, 7.0 and 8.0 respectively).

It seemed from these observations that the stability of the types is a function not only of the purity of the type, but, even more, of the character of the medium upon which the organisms are growing.

While all of the derived types were quite stable, when they were once established, the two intermediate forms showed by far the greatest degree of stability. Strangely enough, however, the rough-intermediate type (Rs) was noticeably more stable than was the normal smooth-intermediate (rS), when this was produced from other definite major types. In several instances there was a repeated swinging back and forth from one type to another as incubation continued, the tendency being toward a reversion to the original type. The cultures seemed to be somewhat more stable on alkaline than on acid media.

Physiological and biochemical properties of parent strains and variants

A comparative study was made of all of the parent strains and the types of variants described above, with regard to their action on gelatin, litmus milk and potato, the reduction of nitrates and the production of indol and hydrogen sulfide.

While there was some difference between different original strains in the type and rapidity of gelatin liquefaction, the variants resembled the respective parent strains in their action, except that the rough and intermediate rough (Rs) variants brought about the liquefaction somewhat more slowly than did the smooth and intermediate smooth (rS) types.

There was considerable difference between the parent strains in their ability to reduce nitrate to nitrite, some being positive and others negative. Corresponding differences were observed in the variants. None of the original strains and variants produced indol, and all failed to show evidence of hydrogen sulphide production.

All of the original strains and variants slowly peptonized the casein of litmus milk, with or without preliminary rennet coagulation. The different variants reacted essentially alike. The litmus was completely reduced in all of the cultures.

On cooked potato the smooth and rough types appeared to differ from each other somewhat during the first forty-eight hours of incubation at 30°C. However, all of the growths showed the characteristic dirty grayish-brown color and pronounced wrinkling after the fourth or fifth day. The potato became progressively more discolored as incubation continued. After ten days it was impossible to observe any difference between the different types.

Growth in extract broth at 30°C. In eighteen hours a faint general turbidity was evident in all of the cultures; a delicate and incomplete pellicle was ordinarily found on the cultures of the R types of all strains at the end of twenty-four hours. Occasionally there were small amounts of flocculent sediment. The S types did not show the production of a pellicle as rapidly as did the R types, but incubation for a period of six or seven days would ordinarily cause the development of a very delicate surface film on the broth. The production of the pellicle was always accompanied by gradual clearing of the broth.

Carbohydrate fermentation. The activity of the various strains was studied in the following carbohydrates, alcohol and glucoside: glucose, levulose, maltose, sucrose, lactose, inulin, mannitol and salicin. The reactions of the variants were practically identical with those of their parent strains. Glucose, levulose, sucrose, inulin and salicin were fermented by all, with distinct acid production. All strains and variants attacked mannitol, but with different degrees of intensity. Some of the original strains appeared to exert a very slight action on maltose, while others were entirely negative. There was little or no difference, however, between the different variants of individual strains. Lactose was not attacked by any of the organisms.

Colony types and cell morphology

While bacterial cell morphology and type of colony generally bear a definite relation to each other, and one would expect, for example, rough colonies to be made up largely of long chains or filaments of the organism in question, there are many notable exceptions, as for example in the long chain-forming streptococci, and in certain single cell variants of *Bacterium pullorum*.

No striking correlation could be established between the types of colony and the length of cells and of chains of *B. mesentericus* and *B. vulgatus*. While there was a greater tendency, on the whole, for the individual bacteria to develop chains and elongated cells in the rough than in the smooth colonies, the difference was not such as to establish a full correlation, in spite of the fact that the rough colonies had the appearance under the low-power lens of containing chains lying parallel to each other, particularly in the irregular border and in runners which extended from one portion of the colony to another (see fig. 4).

FILTRATION EXPERIMENTS

The major interest in the present investigation lay in the possible demonstration of a filtrable stage in the life history of the organisms under study. While many papers have appeared in recent years on so-called filter-passing forms of ordinary bacteria, there seem to be no published claims of such properties for *B. mesentericus* and *B. vulgatus*. Deeply-staining granular material has been observed in the cells of these organisms by various workers. Smeaton and Rettger (1924) observed extreme cellular morphology in a mesentericus-like organism which was isolated from a case of clinical tuberculosis. Granular and bacillary forms of widely different size and shape, which at times were acid-fast, were frequently seen. According to some investigators, there is a direct relation between filtrability and the presence of granules in a culture.

Two different methods of demonstrating filtrable forms were employed: the first was the one used by Hadley, Delves and Klimek (1931) for the production of "G" colonies, in which the organisms were subjected to serial passage through broth containing small amounts of lithium chloride; the second involved exposure of the organism to the action of bacteriophage.

Serial transfer in 0.5 per cent LiCl broth

In the preliminary experiment a large typical rS colony of the F strain was selected from a beef infusion agar plate, suspended in a tube of infusion broth, and the culture incubated for four hours at 30°C. Of this culture, 1 cc. was inoculated into a second tube of broth and the tube incubated for four hours. One cubic centimeter amounts of the last culture were then introduced into each of four tubes of broth, two of plain extract broth (pH 7.6), and two tubes of extract broth containing 0.5 per cent lithium chloride (pH 7.6). Two of these tubes, one of the plain and one of the LiCl broth, were incubated at 22°; the other two were held at 37°. After twenty-four hours, subcultures were made into fresh media, plain sterile broth being inoculated from the plain broth cultures, and LiCl broth from the LiCl tubes. This procedure was repeated serially at intervals of twenty-four hours through seventeen subcultures.

At the time of every culture transfer agar plates were streaked with material from each of the four twenty-four hour broth cultures. Duplicate sets of plates were made: one on plain extract agar, the other on agar to which 0.5 per cent LiCl had been added. Plates made from the 22° cultures were incubated at 22°; those from the 37° cultures, at 37°.

"G-form colonies"

The plates were examined, after twenty-four hours growth, for the presence of colonial variants which might correspond to Hadley's "G-form" colonies. After four or five serial passages, the agar plate cultures of the 22° series showed colonies which were thought to be similar to the "G-forms" described for *B. dysenteriae* (Hadley, Delves and Klimek, 1931). They were very small, often groups of only a few cells being observed which were either barely visible to the naked eye or, as was more often the case, visible only with the aid of a strong hand lens or the low power objective of the microscope.

Microscopic examination of these colonies showed them to be composed of a single flat layer of cells on the surface of the agar. There was not the slightest evidence of a tendency toward heaping-up or dense massing of the cells. Some of the colonies were composed of as few as eight or ten individual cells; others, of a hundred or more. Slide mounts were made of a few of the larger colonies of this type and stained with either methylene blue or the Gram stain. The majority of the cells were fairly uniform rods which were slightly longer and thinner than the normal. Many irregular cells were observed, however. These included "spindle" and "club" forms, in particular. There were also occasional large globular forms which were from three to five times as large as the ordinary cells.

The "G-type" colonies first appeared in the LiCl agar plates which were streaked from the 22° LiCl broth cultures. They were observed on the fifth passage. On the fifth transfer plate very few of the "G" colonies were seen. On each succeeding plate of the series, however, more and more of them appeared until the tenth series, when *only* so-called "G-colonies" were noted. On the eleventh, twelfth and thirteenth plates of the series only this type of colony developed during twenty-four hours incubation; but from the fourteenth series on the number of such colonies steadily diminished until the nineteenth series, when only normal colonies appeared on the plates.

All of the plates which showed "G" colonies at the end of twenty-four hours were held for an additional period of forty-eight hours. In this period a marked change was observed in the character of the growth on the plate. "G" colonies were no longer present. Some of them, the positions of which had been carefully noted, were no longer visible. They seemed to have been autolyzed. Others, which had resembled typical "G" colonies by the end of twenty-four hours incubation had continued development until they were indistinguishable in appearance from the so-called "normal" colonies on the plate. A few "G" colonies were still found on occasional plates after the first forty-eight hours of incubation, but by the end of seventy-two hours all had invariably disappeared.

"G" colonies first appeared on plain agar plates made from the LiCl broth series, on the sixth serial transfer. In the plain broth series "G-forms" first appeared on the LiCl agar plates made from the eighth subculture; on the plain agar plates made from plain broth cultures, on those made from the tenth series. While rapid serial passage in plain extract broth eventually produced "G-form" colonies, the addition of LiCl to the medium induced the development of the "G" character in a much shorter time. "G" colonies were never obtained from the 37° series of subcultures.

For the major experiment of the series, all nine of the original strains of *B. mesentericus* and *B. vulgatus* were seeded into tubes of LiCl broth. These were incubated at 22° and serial transfers and platings made according to the method described above. Growth was normal on all plates through the fourth series, although the colonies were rather small. On the plates of the fifth and sixth series a few scattered "G-form" colonies were observed. On the plates of the tenth and eleventh series, *only* "G" types were apparent at the end of twenty-four hours. Incubation of all plates beyond this period caused the disappearance of the "G" colonies, either by autolysis or transition to the "normal" type.

Filtration experiments with "G-form" colonies

Since Hadley reported that the "G-form" is indicative of the presence of filterable elements in a culture, his methods of demonstrating such a filtrable stage were followed to a certain extent. Numerous "G" colonies were selected from plates of the series. They were removed from the plates with a flattened platinum needle, small chunks of agar on which the colonies rested being cut out, placed in tubes of sterile broth, and thoroughly macerated to free the bacterial cells. These new cultures were incubated at room temperature for varying lengths of time. At stated intervals, they were admitted to a Chamberland L5 candle. Filtrates were held at room temperature and examined at frequent intervals. Also, agar plates were streaked with small amounts of the different filtrates, and material from such plates washed forward according to the method suggested by Hauduroy (1927).

Seventy-two sets of "G" colonies were so treated. Two were selected from each of the plates of the ninth series, three from each of the eleventh series, one from each of the thirteenth, and two from each of the fourteenth.

Of the cultures of the "G" colonies made from the plates of the ninth series, one of the two for each strain was filtered at the end of twenty-four hours incubation. The other was filtered two weeks later. Of those prepared from the eleventh series one set was filtered at the end of twenty-four hours, a second set after two weeks, and the last after two months. All of the "G" colony cultures of the thirteenth series were filtered at the end of twenty-four hours incubation. One set of those from the fourteenth series was filtered after seventy-two hours; the other after two weeks.

One tube of sterile broth was filtered with each set, serving as a control for the whole lot. This gave a total of eighty filtrates to be observed.

All of the eighty filtrates were treated according to Hauduroy's directions, being washed forward with broth from one agar slant to another. (Large agar slants prepared in tubes of one inch bore were used, instead of Petri plates.) In addition, quite frequent platings were made directly from the filtrates. On none of the slants was any growth ever detected. Extensive and irregular drying of the agar surface caused the appearance of many dull, film-like areas on the slants. These were equally frequent on the two series, the culture-filtrate and sterile-broth-filtrate series. Smears of material from these dull patches were entirely lacking in organized cellular elements. No contaminants were found on the agar slants, even when they were held for ten weeks or longer.

A duplicate set of washings was made on Petri plates with seven of the filtrates. The characteristic dulled areas were observed on all of these plates. Contaminants were found on about 3 per cent of the Petri dishes.

Since attempts to demonstrate the presence of filtrable forms by growing them on meat-extract agar had met with failure thus far, media which were believed to be somewhat more favorable for their development were used. The filtrates were streaked on infusion agar and on agar to which 1.5 per cent glucose had been added; they were seeded into glucose broth, into alkaline extract broth (pH 7.6), and into infusion broth. The results were again negative; no filtrable forms could be discerned at any time.

In addition to these filtrates from so-called "G" colony cultures, another set of filtrates was prepared and studied. After the serial subcultures had been made from the thirteenth LiCl broth cultures into the fourteenth set of tubes, and after the usual set of plates had been streaked with material from the cultures, all nine tubes of this thirteenth subculture and one tube of sterile broth were filtered directly. These filtrates were handled in the same manner as the others had been, and all of them proved to be consistently negative.

Incidentally, the plates which had been streaked from these cultures just before filtration showed, after twenty-four hours incubation, only the "G-type" colonies. It was evident, therefore, that at the time of filtration there had been present in the cultures many cells with the potential characteristics of the "G" forms. Since the "G" colonies are supposed, according to Hadley, to indicate the presence of filter-passing elements in a culture, and since so-called "G" forms were present at the time of filtration, it was logical to expect that filtrable forms might be found then, if ever. Many of the filtrates were held for ten months and still showed no evidence of growth.

BACTERIOLYSIS AND FILTRABILITY EXPERIMENTS

Isolation of bacteriophage

Considerable difficulty was experienced in isolating a bacteriophage active against *B. mesentericus* and *B. vulgatus.* Seven different attempts to procure a phage from sewage gave negative results. Water taken from a stagnant pool in which mesentericus-like organisms were abundant apparently did not contain a lytic agent active against any of the nine stock strains used in this investigation.

A *B. anthracis* phage was obtained from a co-worker in the laboratory. He reported this as being active against one of his stock cultures of *B. mesentericus,* but no activity against any of the strains used in this investigation was observed for it. An unsuccessful attempt was made to "adapt" it to the test strains.

By mere chance, all of the negative filtrates, including that of *B. anthracis*, had been held. Some of them had been stored between two and three months; the most recently prepared were three days old. They were all pooled and set up with an equal amount of sterile alkaline extract broth, seeded with a mixture of the nine bacterial strains, and the tubes incubated at 30° for twenty-four hours. A filtrate of this mixture was found to lyse nearly all of the mesentericus and vulgatus strains actively. Lysis was demonstrated in both the filtrate-culture tubes and on agar plates. Numerous plaques were produced in the plates of each of the sixteen cultures tested by this method. The exact source of the phage remains a question. It may have been present in any one or more of the filtrates of the pool sample; it may have been an instance of very slow adaptation of the anthrax phage, or it may be that some factor which previously inhibited the activity of the phages was destroyed during the period of storage. In any event, active lysis of the strains was procured.

Filtration of phage-lysed cultures

The various cultures were subjected to the action of this lytic agent for eighteen to twenty-four hours at 30° and then filtered. The filtrates were held at room temperature for varying lengths of time. They were examined daily; platings were made from them at different intervals. In a series of two hundred such filtrates no growth was observed except in two of the filtrates, which were grossly contaminated.

A fine white precipitate was occasionally found in filtrates which had been held for ten days or longer. This was apparently not composed of living organisms. It produced no growth when inoculated into a series of different media, and in stained preparations it failed to show any organized cellular structure.

Some of the filtrates were carried serially on agar slants, according to Hauduroy's method. Results were consistently negative.

Filtration of cultures exposed to phage action for varying lengths of time

Two sets of cultures were studied in this experiment. In the first, ten separate tubes of alkaline extract broth, each containing 8 cc. of medium, were inoculated with equal amounts of a heavy suspension of the original Am M strain. The amount of inoculum used was just sufficient to yield a faint turbidity in the broth. To nine of the tubes 1 cc. amounts of active bacteriophage were added. The tenth tube was left as a control. The cultures were incubated at 30°. One bacteriophage-containing culture was filtered at the end of every hour during the first eight hours of incubation. The ninth culture and the control were filtered at the end of twenty-four hours. The filtrates were all held at room temperature.

Twenty-four hours after filtration small amounts of the different filtrates were seeded into tubes of meat-extract broth which had been adjusted to pH 5.0, 6.0, 7.0 and 8.0, respectively, into broth containing 1.5 per cent glucose, and onto nutrient agar plates. This set of sixty cultures was held under observation for ten days. All of the broth tubes failed to produce any visible growth.

The American Type Culture Collection strain of *B. vulgatus* was used in the second set of cultures, which was prepared and handled in exactly the same manner as the first had been. The results of these filtrations were also negative, no filter-passing forms of the organism being detected. Many of the filtrates were held for nearly a year and still showed no evidence of growth, when plated out on routine media.

DISCUSSION

In view of the similarity observed in this investigation between *B. mesentericus* and *B. vulgatus,* and of the newer conceptions of bacterial variation, the question may well be raised as to whether these two organisms should not be regarded as one species.

They have essentially the same morphological characteristics, and the cultural reactions observed in the course of this investigation have been quite similar.

The following brief description characterizes both organisms to a very large extent: so-called "normal" cultures of both are made up of rods which are usually 0.5 by 2.0 to 2.7 μ in size. The presence of long, stout cells and occasional chain and filamentous forms may be noted, particularly in cultures containing rough variants. Moderately active motility is common to young cultures of all of the strains. Gelatine is liquefied and milk is peptonized by all. Both *B. mesentericus* and *B. vulgatus* ferment glucose, sucrose, mannitol, inulin, salicin, and levulose, and fail to ferment lactose. Some strains do not ferment maltose, while others do to a slight degree. Nearly all of the so-called "mesentericus" strains failed to reduce nitrates, whereas the "vulgatus" strains exerted a definite reducing action.

The use of the term "mutation" seems scarcely justified, as applied to these organisms. There was a constant tendency for them to vary, but the changes were readily reversible and never strictly "permanent," under the usual cultural conditions.

One of the most interesting points observed in the course of the investigation was the surprising degree of constancy of physiological properties exhibited by all strains, both parent and daughter, in spite of the marked variations in their colony forms. These observations are essentially in accord with those of others who have studied different members of the group (Soule for *B. subtilis*, Nungester for *B. anthracis*, and Gee for his vulgatus-like organism).

From a series of observations made in an attempt to determine the character of the so-called "G" colonies, it seems highly probable that these forms are derived from bacterial cells which are relatively weak and inactive. The organisms are so sluggish that they do not multiply rapidly enough to produce normal, visible colonies within twenty-four hours. The very small colonies which do develop in this period are composed of a few small, almost transparent cells. On prolonged incubation of the "G" colonies either one of two things happens: the small colonies disappear entirely, or they develop into small, but otherwise "normal," colonies. In the first instance, the cells are apparently so weak that they are unable to resist the attack of enzymatic substances present in the culture and are rapidly lysed. In the second, the cells exhibit a somewhat higher degree of vitality and are able to produce "normal" colonies if incubation is continued long enough.

In many of the investigations in which positive filtration results have been reported quite coarse filter-candles were used. It seems probable, also, that in many instances the pores of the filters became coated with materials which tended to neutralize the electric charge on the candle and yet did not clog the pores. A few organisms, especially if they were very small, may have passed through such filters, yielding so-called "filtrable forms." Finally, one cannot refrain from the conclusion that many of the so-called "filtrable forms" were probably contaminants. Refinement of technic should eliminate such in nearly every case.

SUMMARY

Four major types of variants have been described for *B. mesentericus* and *B. vulgatus*. These have been purified and stabilized by serial selection from agar plate colonies through approximately 150 culture generations.

The normal colony type has been designated as a smooth-intermediate or rS type. The extremely smooth variant has been called the smooth or S type. The other two stabilized variants have been called the rough and rough-intermediate, or R and Rs types, respectively.

While considerable variation in colony form has been observed, the organisms have shown a remarkable constancy in their physiological properties.

The stability of the different colony types was studied. The extreme rough and smooth types showed the greatest tendency to be unstable, the R type to an even greater extent than the S. The Rs type, when fully established, seemed in most instances to be somewhat more stable than the rS type. The stability of all types appeared to be markedly influenced by the medium upon which they were growing.

The development of so-called "G" colonies in lithium chloride-containing broth is reported. Attempts to demonstrate that these "G-type" cultures possessed filtrable elements yielded negative results.

The isolation of a bacteriophage which is active against the different strains of both *B. mesentericus* and *B. vulgatus* is discussed. Attempts to produce filter-passing forms of these organisms by exposing them to the action of the bacteriophage proved fruitless.

A true filtrable form was not observed for any of the nine mesentericus-vulgatus strains studied here.

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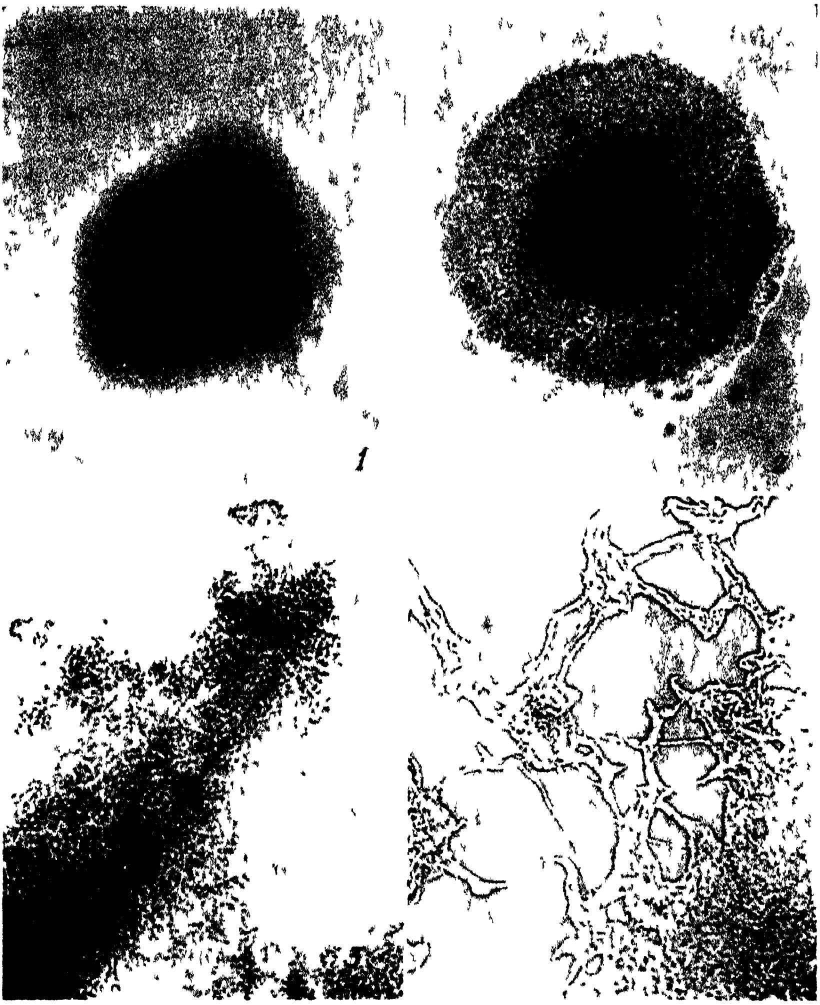
PLATE 1

Fig. 1. *B. mesentericus*, *S* colony on beef infusion agar after twenty-four hours incubation at 37°. × 125.

Fig. 2. *B. mesentericus*, rS colony on beef infusion agar after twenty-four hours incubation at 37°. × 125

Fig. 3. *B. mesentericus*, Rs colony margins on beef infusion agar after twenty-four hours incubation at 37°. × 125.

Fig. 4. *B. mesentericus*, *R* colony margins on beef infusion agar after twenty-four hours incubation at 37°. × 125.



(Catherine S. Flynn and Leo F. Rettger: Studies on B. mesentericus and B. vulgatus)

GROWTH RETARDATION BY FRESHLY DISTILLED WATER

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It has been known for some time that water exists in different molecular forms which influence biological functions in various ways. T. C. Barnes (1932) summarizes his physico-chemical investigations as follows:

> "Water as a liquid is a system of three components; ice, or trihydrol, which is present in greatest concentration at the freezing point; dihydrol, the main component at ordinary temperatures; and monohydrol, or steam, increasing as the temperature rises to the boiling point."

Rao (1933) gives the following approximate compositon of water, in parts per 100:

	ICE	WATER, 0°C.	WATER, 38°C.	WATER, 98°C
Monohydrol, H_2O	0	19	29	36
Dihydrol, $(H_2O)_2$	41	58	50	51
Trihydrol, $(H_2O)_3$	59	23	21	13

Barnes observed that protozoa grow more rapidly in cultures rich in trihydrol than in controls of monohydrol and dihydrol, and that the growth and photosynthesis of Spirogyra is greatly accelerated at 10°C:

> "The working hypothesis is put forward that the colloidal hydrol aggregates are the first building stones in the formation of carbohydrates. The effect on other biological processes appears to be a catalysis phenomenon produced by the colloidal water particles."

Lloyd and Barnes (1932) repeated these experiments under varying conditions, especially with addition of nutritive salts. The result was the same; best growth in melted ice, fair growth in water which had been in the liquid state for many days, and death of most cells in recently condensed steam.

Barnes and Jahn (1933) continued these biological experiments with *Euglena gracilis*, in a culture which received fresh water every day for a period of ten to sixteen days. In the series where the water was obtained from condensed steam, the number of individual cells was increased 32 per cent; with water from melted ice the number increased 105 per cent.

The effect of these different kinds of water upon growth can be studied more accurately if, instead of the slow-growing Euglena, rapidly growing bacteria are used as the test organisms. The results mentioned above can be obtained in 6 hours with *Bacterium coli* as test organism, and a renewal of the water would be unnecessary.

TECHNIQUE

Water high in the trihydrol polymer was prepared by melting ice. Water which contained an excess of monohydrol was obtained by condensing steam. Dihydrol, or normal water, consisted of distilled water which had been allowed to stand for two weeks. The ice was prepared by freezing sterile water blanks and keeping it frozen for at least twenty-four hours. Steam was condensed through a sterile Pyrex condenser. At once the three waters were brought to 30°C., and converted into nutrient broth by the addition of 1 cc. of a sterile concentrate containing 25 per cent peptone and 15 per cent beef extract, to 50 cc. portions of the water. Each of the three broths was inoculated with 1 cc. of a 1:100 dilution of a twelve hour old culture of *Bacterium coli*. Plates were poured every hour for six, then at twelve, twenty-four, and thirty-six hours. The results are given in table 1.

For the first six hours, the number of cells per cc. in the steam water was smaller than in the ice and standard waters. The numbers in both ice and standard waters paralleled each other throughout the experiment. At the end of 25 hours all differences had disappeared.

It seemed possible that lack of oxygen in the condensed steam water might account for the slow growth in media prepared from it, even though the organism is a facultative anaerobe. The above experiment was duplicated, the only difference being that for the first twelve hours, air was bubbled through the three waters at the rate of 1.5 liters per hour. The results corresponded exactly with those of the first experiment, indicating that the effect was not due to lack of oxygen (table 1). Experiment I was repeated at 15°C., giving similar results, but apparently slighter differences.

TABLE 1

Growth of Bacterium coli at 37°C. in standard broth made from different types of water

	CELLS PER CUBIC CENTIMETER DETERMINED BY PLATE COUNT IN		
	Melted ice	Condensed steam	Normal water
Start	40,000	41,750	29,800
After 1 hour	46,000	38,650	44,000
After 2 hours	122,000	69,500	132,500
After 3 hours	645,000	360,000	735,000
After 4 hours	3,650,000	2,145,000	3,400,000
After 5 hours	22,950,000	12,400,000	32,500,000
After 6 hours	63,500,000	40,000,000	61,000,000
After 12 hours	340,000,000	395,000,000	400,000,000
After 25 hours	910,000,000	920,000,000	865,000,000
After 36 hours	1,270,000,000	1,119,000,000	1,110,000,000
With continuous aeration for the first twelve hours			
Start	56,500	54,500	50,500
After 1 hour	109,500	75,500	98,500
After 2 hours	395,000	195,000	301,000
After 3 hours	1,660,000	775,000	1,600,000
After 4 hours	9,300,000	5,255,000	8,650,000
After 5 hours	50,050,000	35,000,000	54,000,000
After 6 hours	170,500,000	101,000,000	163,500,000
After 12 hours	1,295,000,000	775,000,000	1,575,000,000
After 25 hours	1,700,000,000	1,595,000,000	1,820,000,000
After 36 hours	2,120,000,000	2,015,000,000	2,056,000,000

In these experiments not more than ten minutes elapsed between the close of the ice-melting or steam-condensing, and the inoculation with *Bacterium coli.* The question arose as to how long a time is necessary for these waters to reach an equilibrium. Barnes states that the attainment of equilibrium is slow. To determine this time element, the concentrate and bacteria were added to waters which had been in a melted or condensed state for three, four, and twelve hours. In each instance parallel experiments were made with freshly-prepared waters (table 2). Twelve hours is apparently sufficient for the waters to reach equilibrium as the curves paralleled each other throughout (fig. 1).

TABLE 2

Determination of the time required for establishing an equilibrium between the polymers of condensed steam and melted ice

		CELLS OF BACTERIUM COLI PER CUBIC CENTIMETER			
		In broth made from		Controls in broth made from	
		Ice melted for several hours	Steam condensed for several hours	Ice just after melting	Steam just after condensing
Inoculated 3 hours after melting or condensing	Start	88,500	103,500	82,500	94,000
	After 1 hour	113,000	113,000	89,000	94,500
	After 2 hours	225,000	188,000	450,000	200,000
	After 3 hours	1,420,000	700,000	1,400,000	1,225,000
	After 4 hours	12,300,000	5,900,000	6,800,000	5,300,000
	After 5 hours	25,200,000	16,600,000	43,500,000	44,000,000
Inoculated 4 hours after melting or condensing	Start	42,500	37,250	62,000	45,000
	After 1 hour	52,150	48,500	72,500	45,900
	After 2 hours	300,000	176,000	301,500	128,000
	After 3 hours	1,251,800	713,000	1,215,000	615,000
	After 4 hours	9,150,000	4,500,000	7,300,000	2,105,000
	After 5 hours	47,000,000	33,000,000	41,000,000	19,200,000
	After 6 hours	94,500,000	72,000,000	119,000,000	71,500,000
Inoculated 12 hours after melting or condensing	Start	610	575	605	606
	After 1 hour	670	720	665	515
	After 2 hours	1,100	1,200	750	450
	After 3 hours	12,500	12,000	11,500	4,000
	After 5 hours	225,000	215,000	210,000	60,000

This last experiment proves also that the differences are not due to the removal of favorable compounds by distillation. The fresh waters showed the same variation as in table 1. Three and four hours were, however, not sufficient for the establishment of equilibrium.

Even freezing did not change the monohydrol rapidly to the polymers. Distilled water was frozen in a freezing mixture immediately after condensation, and melted again following complete solidification. After adding concentrated food and inoculating there was no difference in the growth rate between the frozen and unfrozen recently distilled water, but both showed a distinctly slower growth than old distilled water (see table 3).

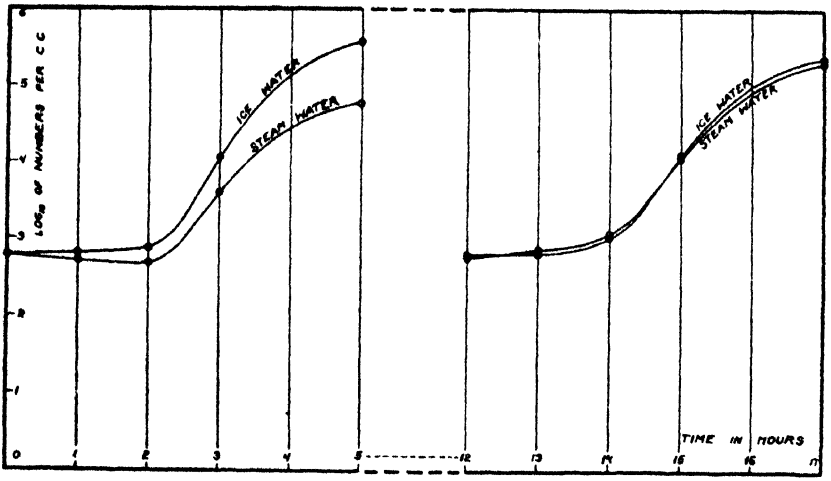

FIG. 1. Growth of *Bacterium coli* in broth made from melted ice or from condensed steam, immediately after melting or condensing, and also 12 hours afterwards.

It has been shown in table 1 that no distinct differences could be obtained between melted ice and standard waters. The bacteriologist is interested in the temperature at which the change into the less favorable monohydrol takes place.

A neutral broth containing 1.0 per cent glucose was prepared. Samples of 50 cc. were respectively frozen, left at room temperature, placed in the Arnold and in the autoclave. Within ten minutes after removal from the sterilizers, all media were brought to 30°C., and inoculated with one cc. of a 1:100 dilution of a twelve-hour-old culture of *Bacterium coli.* Plates were poured every hour for six hours. The growth proceeded at the same rate in all four samples, for the duration of the experiment (table 4). There was no greater variation between the various samples than was found between the ice and standard waters. It appears necessary for the water to be vaporized and condensed to produce a noticeable retardation of growth. Heating alone, for about 20 minutes even to 121°C. is not sufficient.

TABLE 3

Growth of Bacterium coli in melted ice from freshly distilled water

	CELLS OF BACTERIUM COLI PER CUBIC CENTIMETER IN		
	Broth from freshly distilled water, frozen and melted	Broth from freshly distilled water	Broth from normal "old" distilled water
Start....	72,000	69,500	63,000
After 1 hour	99,500	93,000	154,000
After 2 hours....	168,000	168,000	263,000
After 3 hours	835,000	820,000	1,320,000
After 4 hours	4,350,000	4,000,000	6,850,000

TABLE 4

Growth of Bacterium coli in standard broth adjusted to 30°C. after having been heated, or cooled, to various temperatures

	CELLS PER CUBIC CENTIMETER, PLATE COUNT THE BROTH WAS BROUGHT TO 30°C AFTER HAVING BEEN			
	Frozen	Held at room temperature	Heated to 100°C.	Heated to 121°C.
Start	123,000	103,500	91,000	102,000
After 1 hour	130,500	107,000	115,000	133,000
After 2 hours	335,000	285,000	320,000	305,000
After 3 hours	1,610,000	1,225,000	1,445,000	1,485,000
After 4 hours	14,200,000	11,600,000	12,235,000	10,025,000
After 5 hours	67,500,000	47,500,000	48,500,000	49,000,000
After 6 hours	181,000,000	152,000,000	160,000,000	160,000,000

The growth process can be divided into two main functions: the synthesis of the cell constituents from food, and the energy necessary for this synthesis. This energy is obtained by respiration or fermentation which is generally brought about by enzymes.

With bacteria it can easily be determined whether both of the growth functions are affected by the type of water. The effect of water polymers was tested with three different enzymes, catalase, pepsin, and alcoholase.

In the instance of catalase, 1.3 cc. each of molar solutions of KH_2PO_4 and of Na_2HPO_4 were added to 48.7 cc. of the three different kinds of water. Hydrogen peroxide was added to make a 0.02 normal solution. Then 1 cc. of a yeast suspension, consisting of one Fleischmann's yeast cake (12 grams) in 50 cc. of water, was added. At 3, 6, 12, and 30 minutes, 10 cc. samples were blown into H_2SO_4 and titrated with 0.01 normal $KMnO_4$. The flasks containing the waters were kept in chipped ice during the experiment. The rate at which the enzyme destroyed hydrogen peroxide was the same in the three waters (table 5).

TABLE 5

The effect of water polymers upon catalase action

PEROXIDE IN YEAST SUSPENSION	CUBIC CENTIMETERS OF $\frac{n}{100}$ $KMnO_4$ SOLUTION REQUIRED TO DESTROY H_2O_2					
	Melted ice		Condensed steam		Normal water	
	Heated	Living	Heated	Living	Heated	Living
After 3 minutes	28.4	12.6	28.2	12.0	28.2	12.5
After 6 minutes.	28.0	5.4	28.1	6.0	28.1	6.2
After 12 minutes	28.0	1.7	28.1	1.7	28.2	1.8
After 30 minutes. . .	27.9	0.7	28.0	0.5	28.1	0.5

Pepsin was tested by adding 10 cc. of 30 per cent gelatin to 50 cc. of the three waters. One cc. of a 20 per cent pepsin solution (Digestive Ferments Company, U. S. P.; 1:3,000 pepsium) was added, and the speed with which the gelatin was digested was measured by a viscosimeter. There was no difference in the rate of liquefaction in the three waters (table 6).

The alcoholic fermentation in the three waters was tested by means of the fermentometer (Rahn, 1929). To the three types of water, 5 cc. of a yeast suspension, consisting of one Fleischmann's yeast cake in 50 cc. of water, were added. Six per cent glucose was then added. The carbon dioxide pressure produced was measured with a mercury manometer. The differences in pressure were all slight and within the range of experimental error. The experiment was repeated using 2 per cent KH_2PO_4 as a buffer. The pH at the start, ranged from 5.4 to 6.1, but it was 3.7 to 3.8 at the end of the experiment. There was no difference in the carbon dioxide pressure produced. Each of the values in table 7 is the average of at least five determinations made during the first hour after melting or condensing. I and II are duplicate determinations with different fermentometers.

TABLE 6

The effect of water polymers upon pepsin action measured by the viscosity of a gelatin solution

TIME	TIME REQUIRED FOR THE GELATIN-PEPSIN MIXTURE TO BE DISCHARGED FROM A 5 CC. PIPETTE				
	Experiment I		Time	Experiment II	
	Melted ice	Condensed steam		Melted ice	Condensed steam
minutes	*seconds*	*seconds*	*minutes*	*seconds*	*seconds*
Start	60.0	53.5	1	53.0	53.5
4	49.5	50.4	3	50.9	51.2
8	49.0	48.9	5	50.3	49.8
16	48.0	49.0	9	49.0	49.1
30	46.3	47.1	17	48.5	49.0
			31	48.0	47.9

TABLE 7

The influence of water polymers upon the rate of alcoholic fermentation measured by the CO_2-pressure

YEAST SUSPENDED IN	AVERAGE PRESSURE AFTER 6 MINUTES, EXPRESSED IN MILLIMETERS OF MERCURY					
	Melted ice		Condensed steam		Normal water	
	I	II	I	II	I	II
Plain sugar solution......	31.0	30.3	30.0	29.0	33.3	30.7
Sugar + phosphate buffer	34.3	33.2	34.0	31.7	34.9	34.0

DISCUSSION

All of these experiments show that for the growth of *Bacterium coli*, with identical nutrients, freshly distilled water is less favorable than older water. However, distinct growth is observed during the second hour after distillation. This retardation of growth by freshly distilled water can be accounted for by the lack of higher water polymers which seem to be favorable, or necessary, for growth.

Our results with *Bacterium coli* differ in one essential point from the biological effects upon Spirogyra observed by Barnes and associates. They reported a great increase in growth rate by the addition of melted ice (trihydrol) while we could find no differences between growth in recently melted ice, and "old" normal water. This might be explained by the different sources of energy used by the two types of organisms. *Bacterium coli* obtains its energy from the decomposition of organic food by means of enzymes. We could show that three well known enzymes were in no manner affected by water polymers. Spirogyra obtains its energy by the transformation of sunlight into chemical energy. Barnes (see above) believes that photosynthesis is greatly favored by trihydrol.

Though photosynthesis is thus favored by trihydrol, enzyme action shows no such effect. The different growth rates of *Bact. coli* in different waters must therefore be caused by an influence upon the synthetic processes as such. From the table cited above from Rao, it seems most probable that dihydrol is the active agent. The trihydrol content of water is changed greatly by freezing, or by heating to 98°, but neither of these two procedures affects the growth rate. The dihydrol content is not greatly affected by freezing or heating to 98°; it is changed strikingly only by vaporisation which is also the sole process influencing the growth rate of *Bact. coli.*

SUMMARY

The growth rate of *Bacterium coli* in broth made from water recently distilled, is lower than in broth made from ice, recently melted. The growth rate in the ice water is identical to that in water which has been held in the liquid form for two weeks.

This difference between ice and steam waters disappears within twelve hours after the water has assumed the liquid state, but not within four hours.

There is no difference of growth rate in media which have been brought to 30°C. immediately after freezing, or immediately after heating to 100° or 121°C.

The rate of catalase action, of pepsin action, and of alcoholic fermentation is not affected by water polymers.

ACKNOWLEDGMENT

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THE RELATION OF MICROÖRGANISMS TO CAROTENOIDS AND VITAMIN A

I. THE OCCURRENCE OF CAROTENE IN BACTERIA

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Carotene formation has been commonly associated with chlorophyll production because carotene is found in all green plant tissues, where Willstätter and Mieg (1907) have shown that it exists in a definite ratio to the chlorophyll. It is not known to be synthesized by any animals although animal tissues frequently contain carotenoids which have been absorbed from food materials. This has been experimentally demonstrated by Palmer (1922) and others. It is therefore of interest to observe that although bacteria resemble animals in their lack of chlorophyll, they are able to produce this complex plant pigment. An added importance has been attached to this compound since Moore (1929) showed that it can be converted into vitamin A in the animal body.

The presence of carotenoids in bacteria has been suggested by the work of several investigators. Zopf (1889, 1891, 1892, 1893) reported carotenoids with certainty in *Bact. egregium*, *Bact. chrysolgloia*, *Staph. aureus*, *Sphaerolitus roseus*, *Staph. superbus*, *Staph. rhodococcus*, and *Staph. apalitus*. The absorption bands and the solubilities of the pigments extracted from the first three indicate that carotene may have been present. Gurd and Denis (1911) found carotenoids in *Myco. leprae*. Later investigators used Tswett's (1906) chromatographic method with subsequent spectroscopic examination of the various fractions. By means of this technique Reader (1925) identified carotene in *Sar. aurantiaca* and Chargaff (1930) found it in *Myco. phlei* but not in *Sar. lutea* (1932).

Vitamin A has never been demonstrated in bacteria. However, the evidence for its absence is rather unsatisfactory. Assays were made by Wallman and Vagleano (1922), Slanetz (1923), Cunningham (1924) and Bieling (1925) with rats, before the importance of vitamin D for their growth was realized, and since experimental details are not available, it is impossible to check either the adequacy of the basal rations or the quantity of cells used as supplement. In 1932 Skinner and Gunderson reported vitamin A activity in a yellow corynebacterium. They did not determine whether the organism produced vitamin A or some other biologically active substance such as carotene.

It is our purpose in the present paper to give a general survey of the distribution of carotene in bacteria. Proof of the identity of the pigment obtained from several organisms is presented in another paper (1933) in which chemical, spectroscopical and biological data are given.

EXPERIMENTAL

Over one hundred red, yellow and orange species were studied. These were obtained from our departmental stock collections or were isolated from air, water, milk, soil, or sewage and from skin lesions. A detailed description of two of these organisms will be given for purposes of identification and reference. Because of their rapid growth and high carotene content, these organisms have been selected for further study.

The first species to be discussed is the corynebacterium G101 which Skinner and Gunderson had reported to be biologically active as a source of vitamin A. Their findings have been confirmed in our laboratory with the same strain. We have also isolated various cultures, G102, G103, from lake water and these are essentially identical with the Skinner-Gunderson strain. The three cultures were used in determining the species characteristics.

Morphologically, the rods were typically short and plump, but their length varied with age and cultural conditions. A forty-eight-hour culture on nutrient agar contained rods varying in length from 2.5 to 5.0μ and in width from 1.1 to 1.7μ. They occurred singly or in short chains. The ends were rounded. They were non-motile. The Gram reaction changed with age but young cultures were definitely positive. Metachromatic granules were very striking. These disappeared on boiling, were readily stained with dilute methylene blue and were not decolorized by subsequent treatment with 1 per cent sulphuric acid.

The organism grew well on nutrient agar and many other media. Rough and smooth strains were obtained by repeated plating. The rough form gave typical tenacious, rough, granular, uneven, surface colonies, and small, irregular, imbedded colonies; while those of the smooth type were round, smooth and moist on the surface, and small and convex when imbedded in the agar. There was a slight initial turbidity in broth, followed by pellicle formation. Growth was readily initiated from pH 6.2 to pH 8.5. The optimum temperature was 25°C. The organism was strongly aerobic. Early surface growth was a light orange buff (Ridgway 17d) and under favorable conditions this deepened to a good orange (Ridgway 15). In litmus milk no change was observed for about a week, after which the milk became increasingly alkaline and developed an orange pellicle. There was no apparent peptonization. The rough form seemed to initiate growth in milk more rapidly than did the smooth form, although similar differences were not noticed in other media. Gelatin was not liquefied. Catalase was produced in quantity. Growth on potatoes was luxuriant. Arabinose, glucose, glycerol, mannitol, sucrose and xylose were fermented giving a slight acidity but no apparent gas. Galactose, lactose, maltose, and starch were not attacked. Peptones and other complex protein decomposition products were the best sources of nitrogen but fair growth was obtained on aspargin, allantoine, tyrosine, urea, acetamide and nitrates but not on glutamic acid, glycocoll, hippuric acid, piperidine or ammonium salts. It is suggested that this diphtheroid be named *Corynebacterium carotenum* n. sp.

The other organism D202, which will be discussed in detail, proved to be a species of flavobacterium. On nutrient agar after forty-eight hours growth at room temperature, the rods varied in size from 1.8 to 2.5 by 1.0μ. Short chains were common. The rods were motile. They were Gram-negative and exhibited no granules when stained with dilute stains. Growth on nutrient agar was rapid, smooth, moist and spreading. The color changed from a light orange yellow (Ridgway 17) to orange chrome (Ridgway 11) or sometimes to flame scarlet (Ridgway 9). Surface colonies had an opaque, pigmented center with a whitish periphery. Deep colonies were of the pin-point type. Growth was aerobic.

Litmus milk was not changed. Catalase was produced. Gelatin was liquefied. Turbidity, with a tendency to pellicle formation, was observed in broth. In general, growth was better on solid than in liquid media. Growth initiation did not take place above pH 7.8. The optimum temperature was 30°C. A slight acidity was developed on arabinose, galactose, glucose, maltose, sucrose, and xylose but not on glycerol, lactose, mannitol or starch. There was no evidence of gas formation. Complex nitrogenous compounds were required for vigorous growth but nitrates could be utilized. Asparagin, urea, tyrosine, acetamide and ammonium salts were inadequate. This species resembled many, described in the genus Flavobacterium by Bergey (1930), but was not identical with any one type. Since cultures were not available for comparative studies, no attempt was made to name the species.

A sodium-caseinate agar, which prevented the overgrowth of spreaders but allowed the slower growing chromogens to form good colonies, was used in isolating strains from air, water and soil. Other colonies were obtained on nutrient agar. Stock cultures were kept on a peptone-glucose-glycerol agar. As suggested by Conn (1917), the addition of glycerol was often found to stimulate pigment production. The other media listed were used to secure abundant growth for analysis. The 200 grams of potato were chopped up fine, steamed for half an hour in 1 liter of water, and filtered through a fine wire gauze. The yeast-water extract was prepared from starch-free baker's yeast. Ten per cent wet weight was steamed for two to three hours, autoclaved and allowed to settle. Medium 5 was made by infusing the beef over night and then adding the other ingredients. The media were in no sense synthetic, but lipochromes were found only in the peptone. Two synthetic media, made by substituting 5.0 grams of asparagin or 1.0 gram of potassium nitrate for the peptone in the peptone-glucose-glycerol agar, were used to verify the actual synthesis of carotene. Pigment production on these media was maintained for fifteen transfers by four species, viz., D202, D210, G101 and G314.

TABLE 1

	MEDIUM 1—SODIUM CASEINATE 2.0 GRAMS	MEDIUM 2—PEPTONE 5.0 GRAMS	MEDIUM 3—POTATO 200 GRAMS	MEDIUM 4—YEAST WATER 100 CC.	MEDIUM 5—BEEF 0.5 POUNDS
Glucose	1.0 gram	5.0 grams	10.0 grams	10.0 grams	
Glycerol		5.0 grams			50.0 cc.
K_2HPO_4	0.2 gram	1.0 gram		1.0 gram	5.0 grams
$MgSO_4 \cdot 7H_2O$	0.2 gram	0.5 gram		0.5 gram	
NaCl		1.0 gram		1.0 gram	
$FeSO_4$	Trace				
Agar	15.0 grams	15.0 grams	15.0 grams	15.0 grams	15.0 grams
Water	1000 cc.*	1000 cc †	1000 cc.*	900 cc.*	1000 cc.*

* Tap.
† Distilled.

For routine analyses such as were required to obtain the data given in table 2, ten 12-oz. bottles were inoculated with a water suspension of each organism. Each bottle contained about 100 cc. of a suitable solid medium which had been slanted to give the maximum surface for growth. After incubation, the cells were removed with a scraper or by means of a suction pump, and were dried at 37°C. They were then extracted with hot 95 per cent alcohol and the mixture after dilution with water to 85 per cent alcohol content, was shaken up repeatedly with petroleum ether. Partition between these solvents separated the pigment into carotene and xanthophyll fractions. The proportion of pigment in each solvent was estimated with a Lovibond tintometer. The figures given in the table were obtained by multiplying the number of cubic centimeters of solution by the Lovibond *Y* reading, which is an arbitrary number corresponding to a yellow filter, the intensity of which matches that of the solution. Since

TABLE 2

The carotinoid content of certain bacteria

STRAIN	TYPE OF ORGANISM	MEDIUM	COLOR ACCORDING TO RIDGWAY	ALCOHOL FRACTION, CC. × LOVIBOND Y UNITS	ETHER FRACTION, CC × LOVIBOND Y UNITS
B101	*Staph. aureus*	Potato	Orange buff 15	204	95
B102	*Staph. citreus*	Potato	Sulfur yellow 25	204	0
B103	Staph. from milk, W-4*	Beef	Capucine yellow 15	63	0
B104	Staph. from milk, Riddy*	Beef	Maize yellow 19	30	0
B108	Staph. from milk, B-12*	Beef	Apricot yellow 19	60	8
B109	Staph. from milk, 164C*	Beef	Orange buff 15	112	14
B201	*Sar. lutea*	Potato	Picric yellow 23	204	0
A101	Variant from *Rh. meliloti*†	Potato	Empire yellow 21	0	0
A102	Variant from *Rh. meliloti*†	Potato	Morocco red 5	0	0
A103	Variant from *Rh. meliloti*†	Potato	Sulfur yellow 25	0	0
D101	Propionibacterium P52‡	Potato	Grenadine red 7	0	0
D201	Flavobacterium from water	Potato	Aniline yellow 19	900	0
D202	Flavobacterium from water	Potato	Orange rufous 11	3,960	1,270
D203	Flavobacterium from water	Potato	Aniline yellow 19	715	61
D205	Flavobacterium from water	Yeast	Empire yellow 21	10	0
D208	Flavobacterium from water	Potato	Primuline yellow 19	5,200	170
D209	Flavobacterium from water	Potato	Light greenish yellow 25	9	0
D210	Flavobacterium from water	Potato	Orange rufous 11	295	196
D212	Flavobacterium from water	Potato	Lemon chrome 21	2,320	0
D215	Flavobacterium from water	Potato	Antimony yellow 17	520	23
D221	Flavobacterium from water	Potato	Aniline yellow 19	800	4
D304	Bacterium from plant tumor§	Yeast	Deep chrome 17	217	350
D306	*Bact. herbicola***	Potato	Primuline yellow 19	0	0
D407	Short rod from pepper	Potato	Wax yellow 21′	330	0
D502	Short rod from air	Potato	Aniline yellow 19	130	91
D601	Short rod from furuncle	Potato	Amber yellow 21′	780	20
E104	Spore former from water	Potato	Mustard yellow 19′	975	38
E201	*Cl. felsineus***	Potato	Orange rufous 11	0	0
G101	*C. carotenum*††	Potato	Orange 15	1,920	1,470
G102	*C. carotenum* from water	Potato	Orange 15	3,500	4,500
G103	*C. carotenum* from water	Potato	Orange 15	310	315
G105	Hog diphtheroid, not acid-fast‡‡	Beef	Pinkish cinnamon	480	120
G205	"25" semi acid-fast‡‡	Beef	Maize yellow 19	360	360
G206	"97089" semi acid-fast‡‡	Beef	Ochraceous salmon 13′	0	180

TABLE 2—*Concluded*

STRAIN	TYPE OF ORGANISM	MEDIUM	COLOR ACCORDING TO RIDGWAY	ALCOHOL FRACTION, CC. × LOVIBOND Y UNITS	ETHER FRACTION, CC. × LOVIBOND Y UNITS
G208	"49e" semi acid-fast[‡‡]	Beef	Apricot yellow 19	530	525
G209	"33" semi acid-fast[‡‡]	Beef	Ochraceous buff 15′	609	625
G210	"336" semi acid-fast[‡‡]	Beef	Orange buff 15	520	360
G301	*Myco. leprae*[‡‡]	Beef	Cinnamon buff 17″	0	0
G302	*Myco. leprae*[§§]	Beef	Capucine yellow 15	405	288
G310	Columbia acid-fast sap.[‡‡]	Beef	Chamois 19″	250	0
G314	*Myco. phlei*[‡‡]	Beef	Deep chrome 17	529	670

[*] From M. Englebrecht, Wisconsin.
[†] From L. Almon, Wisconsin.
[‡] From E. McCoy, Wisconsin.
[§] From H. Sagen, Wisconsin.
[**] From E. B. Fred, Wisconsin.
[††] From C. E. Skinner, Minnesota.
[‡‡] From J. McCarter, Wisconsin.
[§§] From F. Holford, Wisconsin.

a rather large volume of solution was used, the smaller numbers indicate only traces of yellow. The absolute magnitude of the figures in table 2 is of no significance since the quantity of growth extracted was not constant, but the figures do indicate the relative distribution of pigment in the alcohol and ether layers.

Several generalizations are warranted on the basis of these data. Bacteria of various kinds contain carotenoids including carotene. The lemon yellow strains such as B102, B201, D209, and D212 are lacking in carotene, while this pigment is frequently found in those types which are orange. Excluding the anaerobes, D101 and E201, one observes that a petroleum-ether-soluble pigment was always associated with a definitely orange shade. The acid-fasts and related organisms were particularly high in carotene. No anaerobic organisms were found which synthesized carotenoids, although colored fermentation mixtures of thermophilic cellulose decomposers and of various clostridia were examined. No actinomyces were studied because of the difficulty in obtaining sufficient cells for analysis, but it is quite probable that many of these contain lipochromes including carotene.

The quantity of carotene obtained per gram of cells apparently depends on many factors. The maximum yield from *Corynebacterium carotenum* was 0.35 mgm. per gram of dried cells. The bacterium D304 gave as much as 0.58 mgm. per gram dry weight.

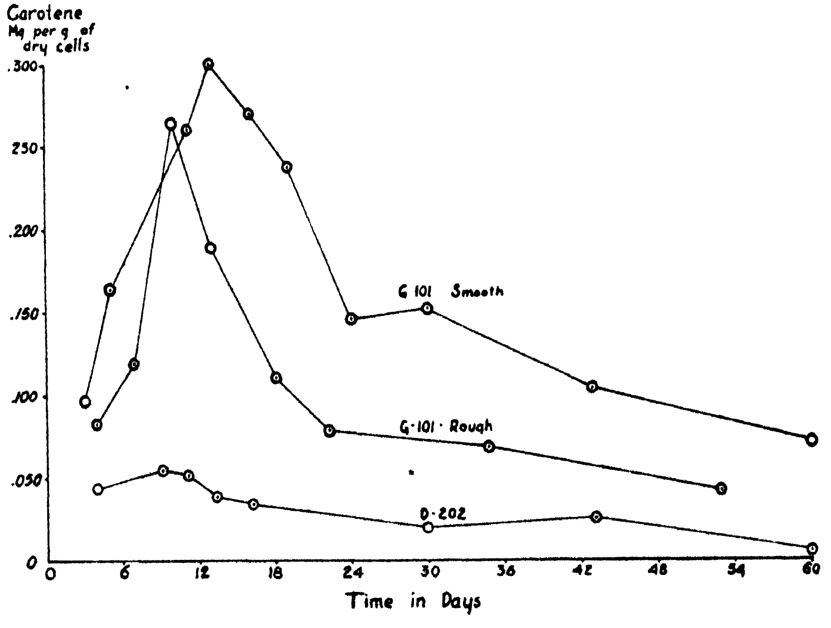

FIG. 1. THE FORMATION AND DESTRUCTION OF CAROTENE BY THREE ORGANISMS GROWN ON A POTATO GLUCOSE MEDIUM

This is approximately twice as much as a carrot contains. The factors influencing carotene production are both biological and chemical. On a given medium the carotene content increases with age to a definite maximum, as is illustrated in figure 1. The tintometer readings for the petroleum-ether fraction were calculated to milligrams of carotene by the equation: $1Y = 0.0005$ mgm. carotene. It will be observed that the rough and smooth forms of the Corynebacterium gave essentially the same curves. Similar results were obtained with G101 and D202 on yeast-water glucose agar and with G314 on Dorset's synthetic medium. Obviously isolated figures such as those in table 2 are but rough indices to the potentialities of the organisms under observation. It remains to correlate carotene production with other growth phenomena.

SUMMARY AND CONCLUSIONS

Although vitamin A has not yet been reported to occur in bacteria, the presence of the biologically active pigment, carotene, has been demonstrated in many organisms. The presence of carotene may be roughly predicted on the basis of the color of the organism. Those of an orange shade generally contain it. No anaerobes were found to synthesize this pigment. The amount of carotene produced by a given organism varies with the medium on which it is grown. The content per gram of cells increases with age to a definite maximum, after which it decreases quite rapidly. A yield of 0.58 mgm. per gram of dry weight was obtained with one organism. The synthesis of carotene by bacteria which was demonstrated on synthetic media, carries interesting biological implications, since these microörganisms do not contain chlorophyll.

No attempt was made here to differentiate between the various carotenes. Investigations of this nature are now in progress.

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AN ORGANISM WITH A TRANSVERSE CAPSULE[1]

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Mucilaginous envelopes, described as capsules, are probably present, if only in small degree, among all bacteria. According to standard texts, in preparations stained in the ordinary manner with basic aniline dyes, the capsule appears in the form of a halo surrounding the bacterial body (Tanner (1928); St. John-Brooks (1930); Park and Williams (1910); et al.). In reviewing the literature relating to bacterial capsules, no mention of defined limited areas of capsule formation could be found. Cotini[2] from information received during a personal interview in 1930, observed a type III pneumococcus having an irregular capsule, the capsule having a relatively greater diameter transversely to the direction of division of the two cells of the diploid; so far as the author is aware, no description of the above mentioned organism has been published.

When studying the bacterial flora of bovine amniotic fluids, an organism was isolated which, when grown on solid culture media such as nutrient or Endo's agar, exhibited a marked viscosity. Colonies were so viscous that when a needle was inserted the culture could be drawn out into fine threads 6 to 10 inches long. Smears, stained by the standard methods for demonstrating capsules, showed the presence of a peculiar type of capsule, the greatest diameter of the capsule being at right angles to the long axis of the bacterial cell. Microscopical and cultural characteristics placed the organism in the genus *Aerobacter*. The observations of the author were verified by Dr. F. C. Harrison, then Professor of Bacteriology at McGill University. The name *Aerobacter transcapsulatus* was suggested and a description of the organism was included in a Master's thesis presented to McGill University (1929).

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[1] Macdonald College Journal Series, No. 38.

[2] I. Cotini, Division of Bacteriology, Institut Pasteur, Paris.

Plate 1 shows the transverse arrangement of the capsule. A description of the organism is as follows:

Aerobacter transcapsulatus nov. sp.

Gram-negative short rod, 0.8 by 3.0μ, single, non-motile.

Growth on gelatin smooth, white, entire, glistening; gelatin stab not liquefied.

Growth on agar plate creamy, viscous, entire, glistening; agar slope white to dirty-white, abundant, smooth, glistening.

Potato slope—growth abundant, white to yellowish white, smooth, glistening.

Litmus milk—acid, milk not coagulated.

Acid and gas in lactose, sucrose, maltose, glucose, galactose, raffinose, rhamnose, xylose, levulose, arabinose, mannitol, adonitol, sorbitol, salicin, dextrin, inositol. Negative in dulcitol and inulin.

Nutrient broth—turbid with white pellicle.

Lead acetate agar—hydrogen sulphide formed.

Voges-Proskauer—positive.

Methyl-red—negative.

Nitrates reduced to nitrites.

Indol not formed.

Blood agar—no hemolysis.

Starch—no hydrolysis.

SUMMARY

An organism showing a transverse arrangement of the capsule has been isolated from bovine amniotic fluid; the cultural and morphological characteristics have been described and the name *Aerobacter transcapsulatus* has been suggested.

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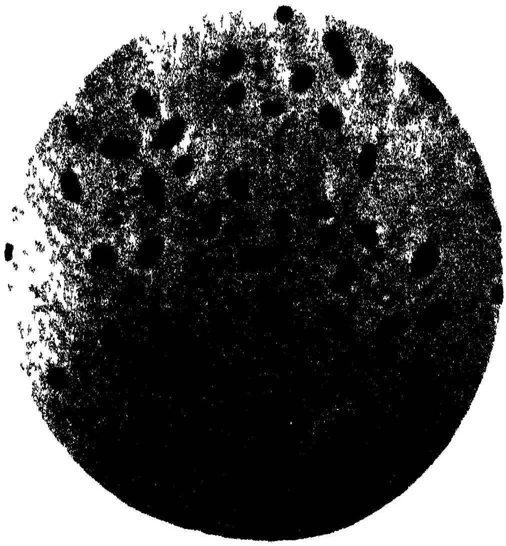

Redvers Thompson Organism with transverse capsule

PLATE 1

AEROBACTER TRANSCAPSULATUS NOV SP

Stained by the Hiss Method (× 2100)

PNEUMOCOCCUS VARIANTS

I. INTERMEDIATE FORMS AND THE INFLUENCE OF ENVIRONMENT IN THEIR PRODUCTION DURING IN-VITRO S TO R AND R TO S TRANSITIONS

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One method of studying the problem of variation in the pneumococcus consists of an enumeration or a classification of the various bacterial "forms" which arise progressively during the process in which S pneumococci lose their ability to elaborate soluble specific substances and R pneumococci gain this ability, or in other words during the degradation of S forms, or the reversion of R forms. This amounts essentially to a series of lines which are drawn more or less arbitrarily through certain involuntary or evolutionary stages of the dissociative process. Such an approach is justified, however, by the fact that Blake and Trask (1923 and 1928) have shown that several, fairly stable pneumococcus variants intermediate between S and R, can be produced with a considerable degree of consistency, and that they are recognizable by characteristic properties. Upon this fact rests the basis of the analysis employed in this communication.

TAXONOMIC LITERATURE DEALING WITH INTERMEDIATE AND R PNEUMOCOCCI[1]

The effort will not be made in the present communication to review literature which deals with the development of knowledge concerning pneumococcus variants. Articles covering this field to which the reader may be referred include those of Kimura, Sukneff and Meyer (1928); Neufeld and Schnitzer (1928); Gundel (1931), and Blake and Trask (1933). It may suffice to say, however, that apart from the recognition of the so-called fixed types of S pneumococci other forms have long been described, which at first were spoken of as "atypical pneumococci" or "streptococci." Later the term rough or R pneumococcus was introduced by Griffith (1923) to cover a large group of variants which have been derived from S types I, II, and III and also from group IV. The term R pneumococcus has been used somewhat loosely perhaps, to designate those variants which produce small, rough-surfaced colonies in contrast to the larger, smooth colonies of the S forms. R pneumococci have also been broadly characterized by avirulence for mice, and loss of the specific polysaccharide to which the "parent" S strains owe their type specificity. They generally retain the ability to be agglutinated by the species-specific fraction of anti-pneumococcus serum, and according to most observers are soluble in bile, although this latter property may be somewhat impaired. That there may be various degrees of stability or "roughness" of the R forms, as expressed in terms of the ease or difficulty with which either spontaneous or induced reversion takes place, has been intimated by several, and this question will be elaborated in the following paper.

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[1] The use of the term R pneumococcus in this paper (as well as the term S) is in accordance with the terminology generally employed during the past decade. Recently Dawson (1933b) has suggested that still further revision of terminology may be necessary, with the term M (mucoid) supplanting the term S; the term S supplanting the term R; and the term R being applied to a new variant which he has described (1933a).

The existence of forms intermediate between S and R has also been described. The main features differentiating the intermediate group are that they produce colonies of which the surface is either smooth, granular, or partially but not wholly rough, and that different forms show characteristic degrees of virulence and agglutination patterns. A classification of this intermediate group, first outlined by Blake and Trask (1923), has been recently published by these authors (1933). As their scheme of classification represents the basis of methods followed in this paper, it will be given in some detail. The intermediate variants described by Blake and Trask were produced by growing S forms (types I and II) in homologous anti-pneumococcus serum broth, and were identified by their colony appearance on rabbit-blood agar plates; their agglutination reactions in homologous and heterologous anti-pneumococcus serum according to the thread test technique, and their virulence for mice. Five variants intermediate between S and R were described which appeared progressively during the degrading process to which a strain of I-S pneumococcus had been subjected. The same general dissociative response was also observed by these authors with II-S strains.

Our own experiences with the application of Blake and Trask's classification and the characteristics whereby we have identified the intermediate variants (designated as I-*a* to I-*e*, inclusive) appear in table 1. Similar characteristics of a I-R strain have also been listed in this table. It will be seen that the major feature in the differentiation of these intermediates has been that of the thread test agglutination reaction based upon various ranges in titre of anti-S agglutination, demonstrable as a firm disc or large flakes; and of anti-P (or anti-R) agglutination, which is quite different and consists of a fine suspension of tiny particles. It should be noted in particular, however, that cross agglutination, *i.e.*, agglutination in low dilutions of heterologous sera first begins to appear in the I-*b* variant; and that complete loss of the coarse flaking (anti-S agglutination) first appears in the I-*c* variant. For the application of Blake and Trask's classification it should also be emphasized that it was devised from stabilized strains, and that the original agglutination reactions were based upon one particular lot of type I anti-pneumococcus serum. Freshly isolated intermediate strains have been often found which present over-lapping properties, and furthermore different lots of anti-pneumococcus serum show a different anti-S and anti-P content, but by and large the classification has proved to be one which may be readily applied and has been used in this laboratory for the past ten years.

A slightly different terminology was subsequently used in Germany by Neufeld and Levinthal (1928), who employed the term -s to designate certain forms intermediate between S and R which were of low virulence for mice or completely avirulent,

TABLE 1

Blake and Trask's classification of pneumococci intermediate between I-S and I-R

PNEUMOCOCCUS	SERUM TYPE	AGGLUTINATION REACTIONS (THREAD TEST)						COLONY		RELATIVE STABILITY OF STRAIN	MOUSE INOCULATION		
		1 20	1 40	1 80	1 160	1 320	1 640	Surface and size	Hemolysis		M L D	Days for M L D to kill	Organism generally recovered
I-S	I	+++ • +•+	+•+ +•+	– + •	– + •	– +	– ±	Smooth and large	No	Quite stable	10^{-7} to 10^{-8}	1–2	I-S
	II	–	–	–	–	–	–						
I-*a*	I	+ +	+•+ +•+	+•+ +•+	++ ++	+ ++	+ +	Smooth slightly smaller than I-S	No	Unstable tends to revert to I-S			
	II	–	–	–	–	–	–						
I-*b*	I	+ ±	+ + •	+•+ + •	+•+ ±	++ ±	+ ±	Smooth slightly smaller than I-*a*	No	Quite stable	10^{-2} to 10^{-4}	2–4	I-*b*
	II	± +	– +	– ±	– –	– –	– –						
I-*c*	I	± ++	± ++	± +	± +	± ±	+ ±	Smooth about ½ size I-S	Slight hemolysis may be present	Quite stable	0.5 cc. to 10^{-2}	3–7	I-S
	II	+ ++	± ++	± +	± +	– ±	– ±						

I-*d*	I	±	±	±	±	±	±	Smooth or slightly granular, very small	Wide zone of hemolysis	Unstable, dissociates to I-*c* and I-*e*	0.5 rarely kills	3	I-S
	II	±	±	±	±	±	±						
I-*e*	I	±	±	±	±	±	±	Smooth or slightly granular, ½ size I-S	Narrow zone of hemolysis	Unstable, dissociates to I-*c*, I-*d* and I-R			
	II	±	±	±	±	±	±						
I-R	I	++ +	++ +	+ −	± −	± −	± −	Rough or granular	Slight hemolysis may be present	Quite stable	0.5 does not kill		
	II	++ +	++ ±	+ −	± −	± −	± −						

Legends:
+++ = firm disk
$\overset{\bullet}{+\bullet+}$ = disk easily broken
$\underset{\bullet}{+}$ = few large flakes

++ = heavy agglutination
+ = fine agglutination
± = very fine agglutination
− = no agglutination

and which gave rise to smooth-surfaced dome-shaped colonies generally smaller than those produced by S forms. Using this terminology as a basis, Klumpen (1932) has recently made an analysis of the intermediates. This author relied essentially upon colony form for his classification. His dissociative patterns were induced by growing S forms in various concentrations of homologous anti-pneumococcus serum, and in media containing animal tissues according to the method of Neufeld and Levinthal (1928). Klumpen used the following terms to designate three intermediate colony types which appeared in the order named: -s colonies, which are described above; -U colonies (Ubergängsformen) characterized by disc-shaped colonies of about the same size as that of an -s with smooth or rough surfaces, in some of which the border was smooth and the center rough; and RK or giant colonies (Riesenkolonien). The latter were very large and frequently had smooth surfaces. They appeared shortly before the true Rs were noted. Other properties of the organisms giving rise to these three types of colonies are described by Klumpen as being unstable. Some were type-specific and virulent, others were not.

Klumpen's -s colonies probably correspond to those produced by Blake and Trask's -*a* and -*b* forms; his -U colonies probably correspond to those of the -*c* forms. We have not had occasion to observe his RK or giant colonies.

GENERAL METHODS

In this communication the terminology employed by Blake and Trask will be followed. The use of the term R pneumococci is restricted to those avirulent forms which give a characteristic R type of thread-test agglutination (see table 1), and produce colonies which have a rough surface, similar to those shown in figures 7 to 9.

Agglutination reactions

These were done in all instances by the so-called thread-test method, in which organisms were grown in a series of six dilutions of homologous and heterologous anti-pneumococcus sera. The method is well known, but as it represents the major basis of our differentiation of the variant groups, a detailed description of the technique employed for testing variants derived from I-S, II-S and III-S strains will be given.

Primarily a series of stock dilutions of anti-pneumococcus sera (types I, II, III)[2] are made up in meat, or yeast extract broth (pH 7.2) in concentrations representing 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640. These are stored in large test tubes and kept in the ice-box. It is of primary importance to use a broth diluent in which fresh strains of S pneumococci will grow without the addition of growth enhancement substances such as blood cells, serum or glucose. We have lately employed for this purpose a modification of a yeast extract broth recommended by Rakieten (1932), which will presently be described.

For each individual test thirteen sterile agglutination tubes (inside diameter 9 to 10 mms.) are employed. The tubes are set up in two rows, a front row of seven, and a back row of six. To the first six tubes of the front row 0.5 cc. of dilutions (1:20 to 1:640) of type I anti-pneumococcus serum are introduced, an equal amount of plain broth is placed in the seventh or control tube. To the back row of 6 tubes similar dilutions of type II anti-pneumococcus serum are introduced. If the organism to be tested is known to be derived from type III, this type of serum dilutions is substituted in the back row. The tubes are then inoculated with one drop from a capillary pipette of a sixteen-hour blood-broth culture of the strain to be tested. They are incubated over night and read the following morning. As is well known the interpretation of this test requires careful controls, for the character of agglutination obtained is dependent upon at least three known factors: (a) the degree of growth, which should neither be too sparse nor too heavy; (b) the relative potency of the particular lot of anti-pneumococcus serum, as different lots seem to differ in their anti-S and anti-P content; and (c) acid agglutination phenomena. The last factor may vary somewhat in different lots of broth depending upon buffer substances and acid producing substances such as glucose. To control the former we have used broth which contains a high concentration of sodium phosphate. The formula for the type of broth which in our hands has during the past year proved satisfactory for use in the thread test may be given as follows:

10 grams yeast extract (Savita)
10 grams "Bacto" peptone[3]
2.5 grams sodium chloride

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[2] I am indebted to the Division of Laboratories, New York State Department of Health, for the anti-pneumococcus sera employed in these experiments.

[3] Since this article was written we have substituted Neopeptone for "Bacto" peptone.

8 grams sodium phosphate ($Na_2HPO_4 \cdot 12\ H_2O$)
1000 cc. H_2O

The Savita, "Bacto" peptone and sodium chloride are dissolved in a liter of water. The reaction is set at pH 7.5 and the sodium phosphate is then added. The medium is autoclaved for fifteen minutes at 15 pounds pressure. It is then filtered through filter paper, bottled in 50 cc. lots and again autoclaved for ten minutes at 10 pounds pressure.

Before any new lot of broth is employed tests should be made as to whether it will yield a good growth when inoculated with virulent and preferably freshly-isolated pneumococci. Subsequently a series of control tests should be set up with organisms of known and characteristic agglutination reactions such as I-S, I-*b*, and I-R.

Colony studies

Individual colonies, representing 36 to 48 hour growths on fresh rabbit-blood agar plates were examined under the low (16 mm.) power objective of the microscope by reflected light from the tilted surface of the plate. As the appearance of pneumococcus colonies is in some measure dependent upon the medium upon which they are grown, it is important that stable control strains of S and R pneumococci should be frequently grown on the same plate for comparison with colonies of the strains to be investigated. In general, plates were streaked to give rise to as many discrete colonies as possible. Particular attention was paid to the surface i.e., smooth, granular, or rough; the size; the configuration, i.e., dome, disk or crater shaped; the outline, i.e., round or irregular; the associated peripheral zone of "greening" of the medium; or a zone of hemolysis; the degree of autolysis, and the development of daughter colonies. In determining colony size at least ten of the largest discrete colonies were selected and their diameter measured by inserting a micrometer eye-piece into the microscope. Several sample colonies representive of those usually encountered in S, intermediate, and R forms appear in figures 1 to 9. In these figures the attempt has been made to show typical and atypical colony appearances, as, for instance, those exhibited by smooth colonies. The surface of the forty-eight-hour S, -*a*, and -*b* colonies is smooth, but when (after twenty-four to seventy-two hours) autolysis has taken place, which is a major characteristic of smooth pneumococcus colonies, a faint pseudo-roughening of the surface may occur (see figs. 2 and 3).

Mouse virulence

The terms *virulence* and *avirulence* have been employed somewhat loosely in the recorded results to designate whether or not 0.5 cc. of an 8- to 16-hour blood broth culture injected intraperitoneally, would kill a mouse. An organism was considered to be avirulent when 0.5 cc. of culture failed to kill within 10 days.

When virulence titrations were done, broth dilutions ranging from 10^{-1} to 10^{-8} were made from an eight-hour glucose blood broth culture, and five mice were inoculated with 0.5 cc. from the five highest dilutions. Plates were poured for colony counts with 0.5 cc. portions of the dilutions higher than 10^{-5}. Unless there were approximately eight or twelve colonies in the plate poured from the 10^{-7} dilution, the result was considered unsatisfactory.

Dissociation of S strains in homologous anti-sera

In our hands this method has proved the easiest and most reliable way of readily producing intermediate and R pneumococci. Representative I-S and II-S strains were grown in broth containing 10 per cent homologous anti-sera. The cultures were transferred daily and at appropriate intervals subcultures were made upon blood agar plates. The colony, the mouse virulence, and the thread-test agglutination of strains picked from single colonies were studied.

Reversion experiments

These were performed by the *in vitro* method described by Dawson (1928), which consists of carrying daily transfers in 10 per cent serum broth using either plain rabbit serum or anti-R serum prepared by the subcutaneous and intravenous inoculation of rabbits with strains of R pneumococci. We are not prepared to say whether anti-R serum broth is actually preferable to plain rabbit serum broth for this purpose. During the first two years in which these experiments were carried out anti-R serum broth was used, and during the latter two years plain rabbit serum broth has been used. Results with the latter method seemed to be as satisfactory as with the former.

Individual forms picked from single plated colonies were studied during the reversion process in the same manner as in the degradation process.

Strains employed

A number of strains of pneumococci were employed in the course of these experiments. They will not be described individually. It may suffice to say that our effort was to test freshly isolated as well as stock S strains. Thus several I-S and II-S strains recently isolated from the blood or sputa of patients suffering from pneumonia or suppurative pneumococcus infections were included; also a highly virulent, stabilized II-S strain which had been subjected to frequent mouse passages over a period of many years;[4] and a number of S, *-a*, and *-c* strains which had been kept on blood agar plates for several months or years. Two stock R strains, I-R and II-R were also constantly used as control strains.[5] Obtained in 1926 they have been kept on blood agar plates with transfers every three or four weeks, and will be described more fully in the following paper.

EXPERIMENTAL

Dissociative patterns in S → R transitions

In order to note the consistency with which intermediates arose in the course of S → R transitions with different S strains, a series of experiments was first run in which daily transfers were made in 10 per cent homologous anti-serum broth. The dissociative response induced in a I-S, two II-S, and a I-*b* form is shown diagrammatically in text-figure 1. Individual variants listed in this figure merely represent the predominant form or forms observed on plated cultures in which agglutination tests and mouse inoculations were done. No attempt has been made to show the relative percentage of two forms when they appeared together. Furthermore, as mentioned above, no attempt has been made to stabilize these individual variants, but merely to classify them by their thread test reactions, virulence and colony type, *as they arose in the course of the dissociative process.* This feature may be in part responsible for the discrepancies which seem to exist between the virulence and the agglutination reaction of some of the *-a* to *-e* forms listed in these experiments when compared with the stabilized *-a* to *-e* strains listed in table 1.

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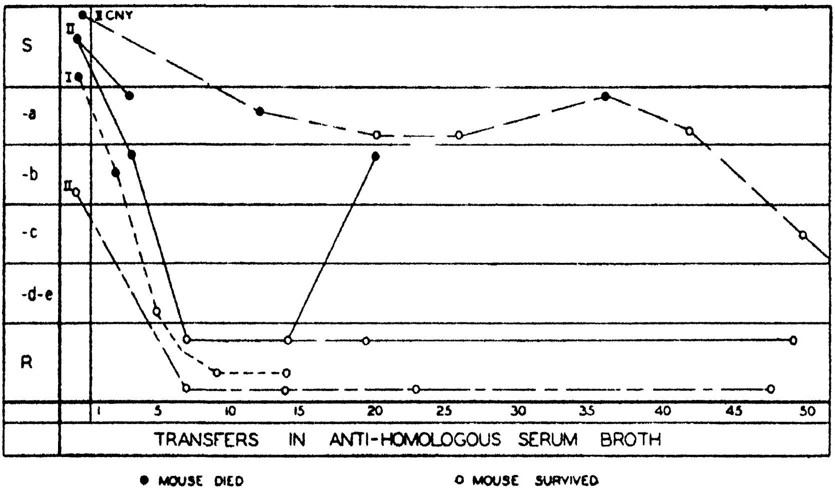
[4] I am indebted to Miss Georgia Cooper of the Bureau of Laboratories, Department of Health, City of New York, for this strain.

[5] I am indebted to Dr. O. T. Avery of The Rockefeller Institute for Medical Research for these strains.

The dissociative responses recorded in text-figure 1 are essentially in accord with those noted by others, namely, the variants successively produced generally follow a fairly rapid progression during their transition to R forms. It will also be noted that there are differences in the rate of dissociative response in some of the experiments. Such differences may be due to unknown variables for it has been our experience that it is difficult to predict, except within wide limits, just what the dissociative response of a given strain will be, even though the identical experiment be repeated several times. On a few occasions not recorded in text-figure 1, we have even noted the rapid transition of S to R forms on the second or third transfers without having previously detected intermediates. There seem to be some measurable variables, however, which influence the dissociative rates induced by this method. These include: (a) differences in the potency of the particular lots of anti-pneumococcus serum employed; and (b) differences in the susceptibility or stability of the individual



TEXT-FIG. 1. Dissociative patterns induced in three S strains, and a II-*b* strain by growth in homologous anti-pneumococcus serum broth. The major trend is that of rapid "degradation" with the production first of intermediate forms and then stabilization of the strain as an R. The response of one of these strains (II CNY) was, however, much slower than that of the others.

strains. For instance the II-S (CNY) strain is an example of the latter effect. It had previously been subjected to mouse passages for a long period (see under Methods) and responded more slowly to the anti-serum. In this strain, although there was a transient period of loss of virulence between the twentieth and thirtieth transfers, virulence was subsequently regained and was present on the thirty-seventh transfer. The fiftieth transfer revealed only avirulent II-*c* forms.

It further appears in these and other similar experiments that the end stage in the degradation process induced by this method, was essentially that of the R form. In a single instance, not shown in text-figure 1, we have carried a strain in which R forms appeared on the twelfth transfer, to the eighty-fifth transfer and it still yielded only typical R forms with an occasional spontaneous throw-back to a virulent intermediate form such as that recorded in the twentieth transfer of strain II-S.

Dissociation of S Strains in Media Containing Bile

It is recognized that if S pneumococci are grown in successive transfers of broth originally containing small but increasing amounts of bile, dissociation to R forms may be induced. This procedure has been used by Reimann (1925), who employed the method of rapidly transferring S cultures in broth containing a 1:400 dilution of bile, later 1:200, and finally after 69 transfers, growth was maintained in broth containing 75 per cent of bile.

Methods of growing S pneumococci in bile

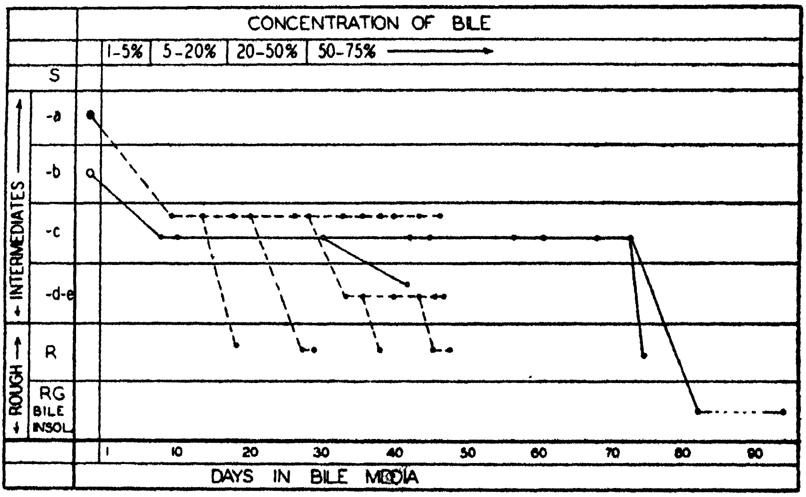
In our attempts to bring about variant production through growth in bile, difficulty was frequently encountered in maintaining the viability of cultures which were successively transferred, so we resorted to growing pneumococci in a continuous flow of broth to which bile was gradually added.

The continuous culture apparatus was a modification of that described by Felton and Dougherty (1924), in which the major alteration was that the rate of flow of medium was adjusted by a screw clamp instead of an automatic electrical device. The apparatus was adapted for use in an ordinary incubator in which holes were bored in the top and sides to allow for entrance and exit tubes. Meat extract broth (pH 7.2) was employed, to which measured amounts of ox bile (which had been sterilized in the autoclave) were added. The culture was maintained in a U-tube of 100 cc. capacity. At the beginning of each experiment the apparatus was filled with broth from a 2-liter graduated reservoir bottle and the U-tube was then inoculated with 1 cc. of a fresh blood broth culture of the strain to be tested, together with a few drops of fresh rabbit blood for the initiation of growth. The rate of flow was then adjusted so that initially between 75 to 100 cc. of media passed through the apparatus every twenty-four hours. Measured amounts of bile were then slowly added to the reservoir bottle from a graduated cylinder of 500 cc. capacity. Daily readings of the rate of flow through the apparatus were recorded together with a record of the increasing per cent of bile in the reservoir. Cultures, taken from a sampling tube, were made at intervals of a few days and the characteristics of the organisms thus isolated were studied.

It was found that a culture of S pneumococci inoculated into a continuous flow of medium could survive the rapid addition of far higher concentrations of bile than we had been able to use in our preliminary experiments of transferring the cultures from tube to tube. Provided a rapid rate of flow was at first maintained, this concentration could be increased to from 1 to 5 per cent within the course of a few days and subsequently to much higher concentrations. Eventually the flow could be diminished or stopped for many days at a time. Another feature which should be mentioned is that when medium containing bile reached the U-tube of the continuous culture apparatus the usual evidences of bacterial growth disappeared, and the medium became quite clear with a brownish sediment at the bottom of the tube. However, samples of this relatively clear fluid yielded growth of pneumococci on blood plates. The period in which cultures of pneumococci would remain viable in this apparatus was largely dependent upon the rate at which fresh medium was added. Our usual experiment lasted from fifty to one hundred days. Occasionally towards the end of an experiment, when very few colonies appeared on the plates inoculated from the sampling tube, cultures on the next one or two days would yield no growth, but on the subsequent day or days, a few colonies would again be found. When cultures remained sterile for more than a week the experiment was terminated.

Dissociative patterns obtained in bile

The object of these experiments was to compare the dissociation of S strains with that induced by growth in anti-pneumococcus serum broth. Six experiments were carried out in which cultures were maintained in a continuous flow of medium containing bile for from fifty to one hundred days. The results of two of these experiments appear in text-figure 2. The dissociative patterns will not be described in detail for in general the "degradation" process induced, resembled that brought about by antipneumococcus serum, but the S → R transition seemed to take place more slowly, and the major trend was for the organism to become more or less stabilized as intermediate *-c* or *-d* forms, rather than as R forms. Many R forms were, however, produced generally after twenty or thirty days; most of them were partially bile insoluble, a few bile insoluble. Furthermore, in the latter part of one experiment (see text-figure 2) a bile insoluble strain was isolated which produced very rough colonies and a granular growth in broth. This type of variant, which will be discussed in the following publication, has been designated as an RG (bile insoluble form). It was only observed on two occasions at the termination of one experiment, the other five experiments were terminated by the death of the dissociating strain.



TEXT-FIG. 2. Dissociative patterns induced in two strains (I-*a* and II-*b*) in media containing bile. The I-a dissociants are shown as small dots, the II-b dissociants as small circles. The major trend is that of stabilization as *-c* and *-d* forms with the occasional production of R forms, and rarely bile insoluble so-called RG forms.

TABLE 2

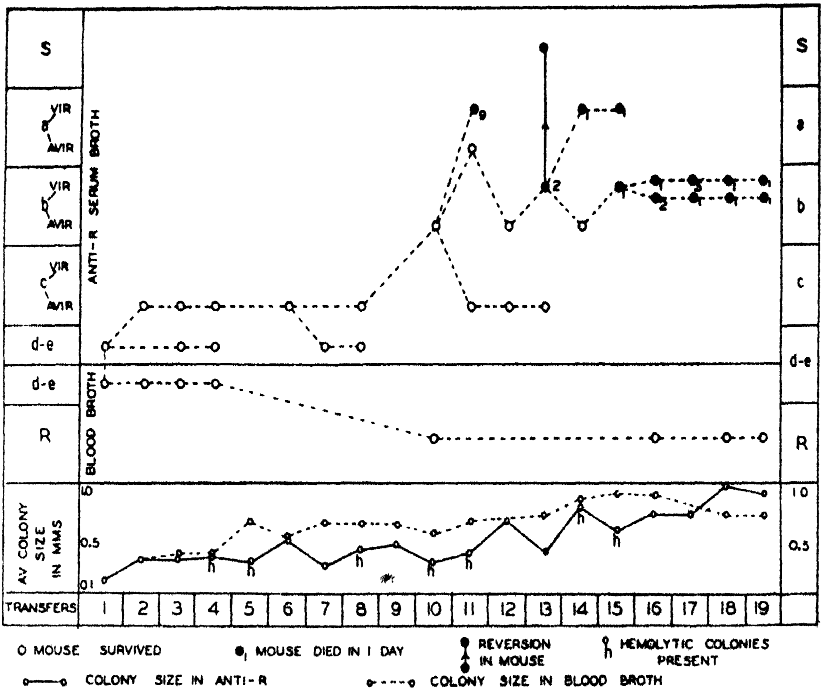
Agglutination reactions and other characteristics of forms arising during in vitro reversion of I-R → I-b

TRANSFER	ORGANISM FROM ANTI-R SERUM BROTH								MOUSE INOCULATION	ORGANISM FROM MOUSE					
	Colony surface	Serum type	Agglutination reactions					Form		Agglutination reactions					Form
			1:20	1:40	1:80	1:160	1:320			1:20	1:40	1:80	1:160	1:320	
1	Rough	I	+	+	±	±	±	I-R	Survived						
		II	±	±	±	–	–								
4	Granular (smooth?)	I	+	+	+	+	±	I-*c*?							
		II	±	+	+	±	±								
8	Smooth	I	+	+	±	±	–	I-*c*	Survived						
		II	±	±	±	–	–								
12	Smooth (hemolytic)	I	+	+	+	+	±	I-*c*							
		II	±	±	±	±	–								
16	Smooth	I	+	+	+	+	±	I-*c*	Survived						
		II	+	+	+	±	±								
20	Smooth	I	+	+	+	+•+	+•	I-*b*	Died 4 days	+	+	+•+	+•+	+•+	I-*b*
		II	–	±	±	±	–			–	–	–	±	–	
24	Smooth	I	+	+	+	+•+	+•+	I-*b*	Died 3 days	+	++	+•+	+•+	+•	I-*a*
		II	±	+	+	+	±			–	–	–	–	–	

Legends as in table 1.

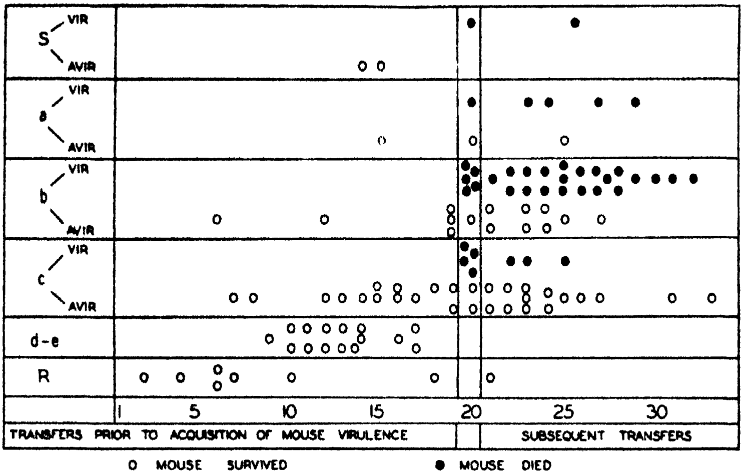
In vitro reversion patterns of R and intermediate forms

In the reversion process induced by the growth of -*c*, -*d*, or R forms in 10 per cent anti-R serum broth or in plain rabbit serum broth, a fairly consistent pattern also resulted. Some R strains proved refractory and failed to show any change after one hundred or more transfers in this environment. When reversion was induced, however, intermediate variants were regularly produced in the reverse order from that in which they appear in the S → R transitions. All of the strains reverted by this method went back to the same type from which they had been originally derived. A single experiment in which reversion of a I-R to a I-*b* was induced by growth in anti-R serum broth, is shown in table 2. A single colony, representative of the pre-



TEXT-FIG. 3. Dissociative patterns of a II-*d* strain in two different types of media. In anti-R serum broth reversion occurs in which the eventual trend is that of stabilization as a II-*b*; in blood broth the strain is stabilized as a II-R. The gradual increase in the average colony size in both of these environments is shown by graphs in the lower portion of the figure.

dominant form on the plated cultures from individual serum broth transfers, was picked daily and from this culture a mouse was inoculated and the agglutination reaction was determined. It will be seen that by the eighth transfer the colonies had become definitely smooth I-*c* forms, maintaining this form until the twentieth transfer when a I-*b* type of agglutination was first noted and the organism had also acquired the ability to kill a mouse in four days. On the twenty-fourth transfer reversion from I-*b* to I-*a* took place as a result of the mouse passage.



TEXT-FIG. 4. Composite reversion patterns in a series of eleven strains originally representing -R, -*d*, and -*c* forms, induced by growth in anti-R serum broth and in plain rabbit serum broth.

A similar experiment is shown in text-figure 3. Here the original strain, a II-*d*, was carried for twenty transfers in anti-R serum broth and also simultaneously in blood broth. Two colonies were picked each day for study. The diagram in text-figure 3 illustrates the apparent complexity of the dissociative pattern in which this labile strain became stabilized in two different environments, in one of which partial reversion took place, and in the other slight degradation. With the former, or in anti-R serum broth, the colonies gradually became larger and smoother and by the eleventh transfer organisms from one colony, which gave a II-*a* type of agglutination, had acquired the ability to kill a mouse in nine days. II-*a* forms were again found on the fourteenth and fifteenth transfers, but eventually the culture became more or less stabilized as a II-*b* maintaining this form from the fifteenth to the nineteenth transfer. The virulence of a culture picked from a colony on the nineteenth transfer was found to be 10^{-4}

It is of some importance to note that the only II-*b* form which reverted to a higher form in a mouse was among the first of the II-*b* forms to appear. This recalls a feature which has been noted by Blake and Trask (1933), namely, that when forms of low virulence (particularly -*c* forms) kill mice, it generally takes three to seven days for the mouse to die but the organism recovered from the dead mouse is usually found to be an S or an -*a* of high virulence. On the other hand, -*b* variants kill mice more readily and more quickly but do not show as much tendency to revert to a higher form during the process.

Conversely, in the blood broth transfers the original II-*d* strain gave colonies which also gradually increased in size, but became rougher instead of smoother. By the tenth transfer only R forms were found. This stabilized R strain subsequently failed to revert when carried for twenty transfers in the same anti-R broth in which reversion had been induced in its original parent II-*d* strain.

A similar trend induced by *in vitro* methods with a series of eleven strains representing -R, -*d*, and -*c* forms is shown in text-figure 4. In charting these results only those forms isolated *in vitro* have been recorded, and no attempt has been made to trace the dissociative patterns of each individual experiment. It will be seen from text-figure 4 that the major reversion trend is again in the direction of -*b* forms. Prior to the first acquisition of mouse virulence occasional forms, probably unstable, appear which give -S or -*a* types of agglutination. Subsequent to the first acquisition of mouse virulence, most of the S and -*a* forms maintain their virulence, but on the whole the organisms tend to stabilize themselves at the level of -*b* forms and not as the typical S and -*a* forms which we are accustomed to find in human or animal infections by the pneumococcus.

Another feature not very well shown in text-figures 3 and 4, is that when reversion of R, *-d* or *-c* forms takes place in mice; this *in vivo* reversion is apt to represent a rapid "ascent" to an S or *-a* form and whether intermediates actually appear in the mouse during the reversion process is not known. At least they were not isolated in these experiments although a careful search for them was not made.

COMMENT

Emphasis has been laid upon conditions which give rise to the development of forms intermediate between S and R pneumococci. These forms probably represent organisms endowed with varying amounts of S substances, or potentialities to develop S substances. They may appear progressively in the S → R transitions, and in reverse order in the R → S transitions described in this paper. Although symbols, based on priority of usage, have been employed to describe phases of the dissociative patterns, it has not been the major aim of this paper to classify rigidly the variants which have arisen during the evolutionary processes described. We have attempted rather to illustrate certain biological trends which occur in the pneumococcus species under certain conditions. Furthermore as the nature of these dissociative processes or trends is probably complex, still relatively obscure, involving as it does certain unsolved problems concerning so-called bacterial life cycles, and as we cannot evaluate the influence which the hypothetical presence of bacteriophage may have played, the attempt will not be made to discuss the mechanism of the processes involved. It would seem, nevertheless, as if the dissociative patterns above described have been limited, or influenced to some extent by the environment in which they have been produced. Our interpretation of this latter phenomenon is that the pneumococcus seems to emerge from a given environment in the form most fitted to survive in that environment. This concept renders a somewhat philosophical value to the question as to what form actually represents the true parent strain of the pneumococcus species, and this point will be elaborated in the following paper.

SUMMARY

1. The importance of recognizing intermediate forms which occur during *in vitro* transitions of S → R and R → S pneumococci has been stressed as a method for the study of dissociative processes in this species.

2. Two different methods of inducing degradative dissociation in S forms seem to give rise to two different patterns of variant production. Thus when S forms are grown in homologous antisera they become rapidly stabilized as R forms, but when S forms are grown in media containing bile, they show a greater tendency to become stabilized as *-c* forms.

3. During reversions of *-c*, *-d* and R forms, induced by growth in anti-R or plain rabbit serum broth, intermediates have been shown to arise in the reverse order to that in which they arise during the degradation of S forms, although such strains tend to become stabilized as *-b* forms, which is the usual "high" level to which these strains revert by this method.

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PLATE

PLATE 1

The colonies shown in figures 1 to 9 are 36- to 48-hours old unless otherwise specified. All but figures 1 and 7 represent a magnification of about 25 diameters.

FIG. 1. II-S colony (×20), dome shaped with smooth surface. Autolysis has not yet begun.

FIG. 2. I-*a* colonies. Flat disc shaped colonies in which autolysis has begun. The surface is faintly granular.

FIG. 3. II-*b* colony. After incubation for forty-eight hours this colony was left at room temperature for ten days. Some autolysis has taken place. The surface is faintly granular.

FIG. 4. II-*c* colonies. They are disc shaped, the surface is almost smooth, but granular about the periphery. The slightly elevated center is often a characteristic of intermediate and rough colonies.

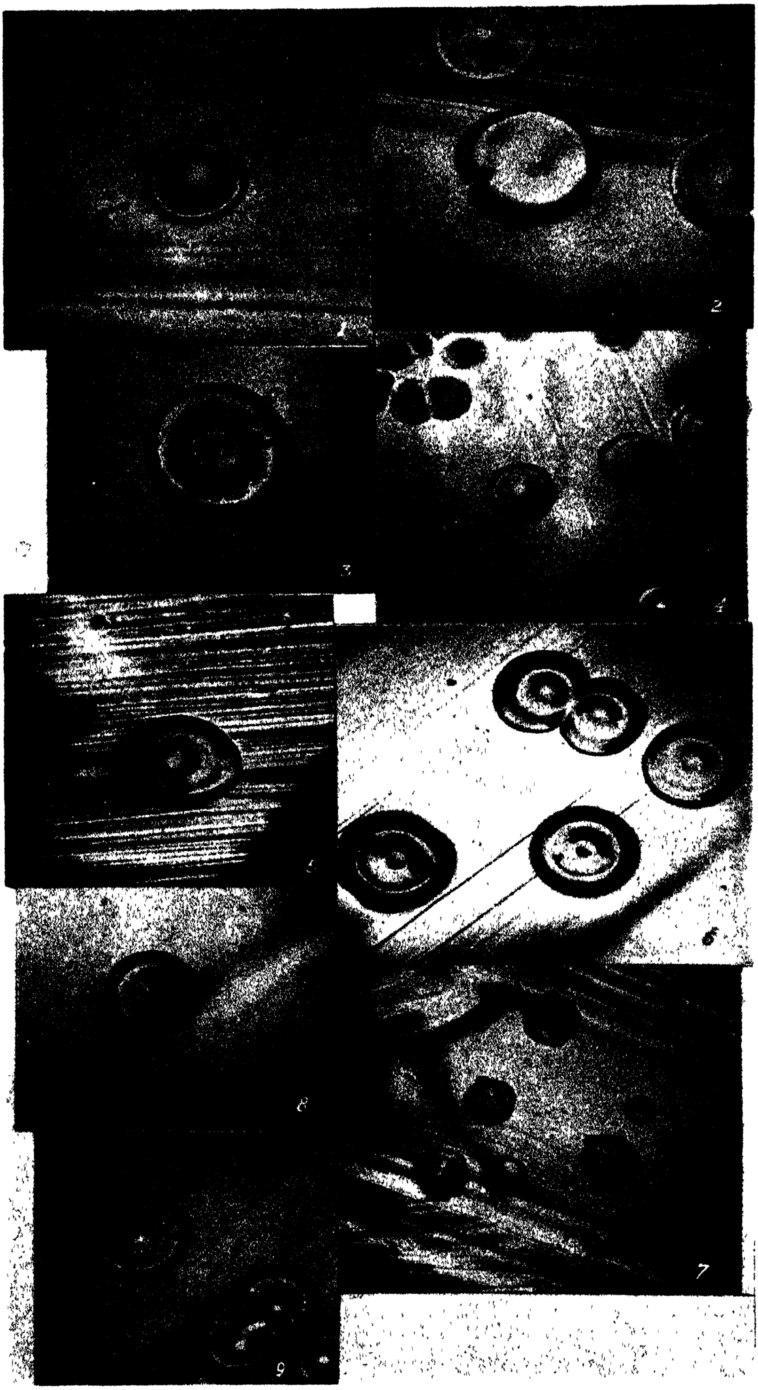
FIG. 5. A II-*c* colony. The surface is fairly smooth; slight central autolysis is present.

FIG. 6. II-*c* colonies.

FIG. 7. A mixture of II-*d* and II-R colonies (×15), representing a strain dissociating in media containing bile. The colonies were grown for 48 hours in the incubator and then stood for three days at room temperature. The II-*d* colonies did not autolyze. The II-R colonies have become irregular with daughter colony formation.

FIG. 8. A rather typical II-R colony.

FIG. 9. II-R colonies.



(John R Paul: Pneumococcus variants)

PNEUMOCOCCUS VARIANTS

II. DISSOCIANTS OF R PNEUMOCOCCI AND THEIR RELATION TO STREPTOCOCCUS VIRIDANS

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INTRODUCTION

In the preceding article pneumococcus variants intermediate between S and R forms have been discussed and a comparison has been drawn between dissociative patterns exhibited during in vitro S → R, and R → S transitions. The present communication will deal with that heterogeneous group of organisms known as R pneumococci and their dissociation, with particular reference to bile-insoluble R forms, or "streptococcus-like" forms which have previously been described according to the latter terminology by many authors.

Review of literature. The literature concerned with observations presented in this paper will be approached from two angles; that dealing with: (I) the transformation of S pneumococci into "streptococci"; and (II) the properties of R pneumococci and their variants. These two avenues of approach are mutually related, for when R pneumococci became recognized it was felt by many that this discovery reconciled the claims of those who described the transformation of pneumococci into "streptococci," and the question would resolve itself into one of definition, or of nomenclature. Indeed, in some instances one could be accused of quibbling as to whether the term R pneumococcus or streptococcus is preferable, for a growing literature bears witness to the fact that the actual differences between some R pneumococci and streptococci are occasionally so slight as to reach the vanishing point. Particularly is this true of the bile solubility test, which has often represented the criterion upon which the differentiation hinges, although it is difficult to express the results of this test in sharply defined terms inasmuch as bile-resistant R pneumococci have been described by Reimann (1927), and by Neufeld and Levinthal (1928), and indeed there are instances of R pneumococci which are almost completely bile insoluble such as those reported by Downie, Stent, and White (1931), and also in the preceding paper.

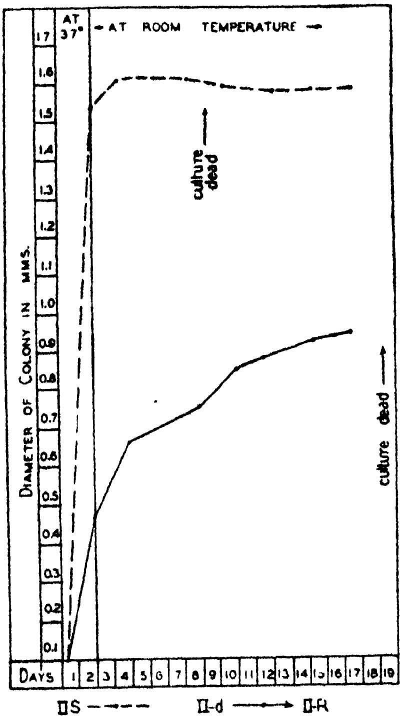
I. The literature upon the transformation of S pneumococci into "streptococci" is extensive and somewhat controversial, but most of the early work which antedated the use of the term R pneumococci has been covered by reviews on the subject of pneumococcus dissociation by Neufeld and Schnitzer (1928), and Gundel (1931), to which the reader may be referred. Of the early observations those of Rosenow (1910 and 1914) are perhaps the best known, and describe changes occurring in strains of pneumococci whereby the organism assumed the appearance of both *alpha* and *beta* streptococci. Subsequently there were several similar reports. It may be pertinent to point out, however, that somewhat in contrast to the systematic studies which have been done upon the S → R pneumococcus transitions in homologous anti-sera, the "transformation" studies have involved a variety of in vitro and in vivo methods, none of which has seemed to give rise to sufficiently consistent results to have been later employed as a standard method by several investigators; in other words, the use of a variety of methods has given rise to a variety of results.

A well known study of this type is that of Morgenroth and his collaborators (1925), who described in vitro changes which occurred during the growth of pneumococci in broth containing optochin, resulting in the gradual transformation of pneumococci into "streptococci." Three stages of this process were described: modification A, in which the organism retained most of the characteristics of pneumococci; modification B, representing bile-insoluble forms which were considered identical with *Streptococcus* *viridans*; and modification C, which was only occasionally obtained, and corresponded to *Streptococcus hemolyticus*. Modifications A and B were also obtained by Schnitzer and Berger (1924), and Berger and Jakub (1925), from mice which had been injected with S pneumococci several hours previously. Heim and Schlirf (1926) could not substantiate the earlier experiments of Morgenroth et al., holding that the supposed variants were contaminants. Silberstein (1927) on the other hand, confirmed the findings. He differentiated R pneumococci from modification B, on the basis of bile insolubility, and described the transformation of a single-cell culture representing modification C, originally derived from a group IV-S pneumococcus, back to a I-S. Reimann (1927a) and Klumpen (1932) have also repeated these experiments. Both were able to induce some dissociation of S forms by Morgenroth's optochin method. Reimann believed that the variants thus obtained were not streptococci but R pneumococci, because they were bile resistant rather than bile insoluble. Klumpen was only able to obtain pneumococcus variants of the intermediate type.

Subsequently several reports largely emanating from Germany, describe the production of *Streptococcus viridans* or *Str. hemolyticus*, both by in vitro or in vivo methods, and their reversion by the latter method. Many of these have been summarized in Neufeld and Schnitzer's review, and the attempt will not be made to transcribe them because of the difficulty of interpreting the findings, resting as it does largely upon the difficulty of defining a streptococcus; for, although some representatives of *beta* or *hemolytic* streptococci would seem to be fairly specific organisms, the *alpha* or *viridans* group is represented by organisms which are far from having specific or fixed properties. It seems unfortunate that many of these investigators failed to compare the properties of their "streptococci" with those of R pneumococci, but it may suffice to say that some of these streptococcus-like forms simulate the R variants which may be derived from the growth of S forms in homologous anti-sera, whereas others do not. In the interpretation of this evidence it is pertinent to recall that the limits of pneumococcus dissociation are not yet known, and the fact that certain suspected avirulent variants do not conform to those produced by the growth of S forms in homologous anti-serum does not exclude them from being possible derivatives of the pneumococcus species.

II. In turning to the literature dealing with R pneumococci the attempt will not be made to review the development of knowledge of the dissociation of S to R pneumococci, for, as also mentioned in the previous article, there are several reviews of this subject such as those of Reimann (1925); Neufeld and Schnitzer (1928); Kimura, Sukneff and Meyer (1928), and Gundel (1931). Although there is relatively little disagreement about the general properties of R pneumococci which have been produced by growing S pneumococci in homologous anti-serum, the term R pneumococcus has, however, often been somewhat loosely applied. Questions with regard to the use of the term have largely arisen in connection with those variants produced by methods other than the one just mentioned, and it is upon this point that the discussion in the present article will hinge.

At the risk of becoming over-exacting with regard to nomenclature it seems important at the outset to distinguish R pneumococci from the forms intermediate between S and R, which have been discussed in the preceding article. R pneumococci derived by degradation of S forms in homologous anti-serum broth show in general an exaggeration of the properties which differentiate the intermediates from the S forms. They seem to be representatives of a hardier race as far as artificial media are concerned, than that exhibited by their more fastidious and more pathogenic parent strains. A summary of the colony characteristic of the R forms has been made in two publications, Paul (1927a and 1934), in one of which (1927a) the similarity between R pneumococcus colonies and those produced by *Streptococcus viridans* has been emphasized. Attention has also been called to the size of the rough pneumococcus colony, which, although variable, is generally smaller than that produced by S, *-a*, or *-b* forms; its surface, which is distinctly rough rather than granular; and its shape, which is apt to be that of an irregular dome. Other colony characteristics include the failure of the R colony to undergo autolysis on aging, in the characteristic manner of the S forms; and the occasional presence of a peripheral zone of hemolysis. This latter feature (also often seen with colonies of the intermediate group), is a point which has probably given rise to some confusion in the past, for such forms may possibly have been designated as "hemolytic streptococci."



TEXT-FIG. 1. CURVES SHOWING THE RATE OF INCREASE IN COLONY SIZE EXHIBITED BY A II-S COLONY AND ITS II-*d* VARIANT

After fifteen days at room temperature R forms were isolated from the II-*d* colony.

Illustrations of typical R pneumococcus colonies appear in figure 1.

Morphologically R pneumococci generally stain as small, Gram-positive diplococci which may appear in chains when grown in fluid media. Capsules are not demonstrable. Reimann (1925) has described R pneumococci as varying in size and morphology and showing bizarre, bacillary and irregular forms.

The character of the growth of R pneumococci in various types of broth is one which does not seem to have been often described. It has been somewhat taken for granted that the majority of typical strains give rise to a diffuse growth similar to that of S forms in plain, glucose or rabbit blood broth. On the other hand, of eight I-R and II-R strains described by Kimura, Sukneff and Meyer (1928), which had been derived from S cultures in homologous anti-sera, four grew granularly in serum bouillon.[1] Neufeld and Levinthal (1928) described R forms produced by growing S pneumococci in media containing sterile organ extracts which gave a granular growth in bouillon.

Agglutination reactions exhibited by R pneumococci with type-specific anti-pneumococcus sera are fairly well recognized and have been discussed in the preceding communication. R forms contain the nucleoprotein antigen common to the species, but not the polysaccharide antigen characteristic of the type from which they were derived. In terms of the thread-test reaction they fail to show agglutination by the type specific (anti-S) fractions of anti-pneumococcus serum, but are agglutinated by the species specific (anti-P) fraction. Reimann (1926) has shown that anti-sera prepared with R forms are immunologically similar to anti-sera prepared with the protein of the pneumococcus. With the thread test, sera of Types I, II or III, prepared by the New York State Department of Health, show an anti-P titer which may run out to 1/10,240. Different lots of sera vary considerably in this respect, however, depending upon their anti-P content.

The property of bile solubility of R pneumococci is one to which considerable attention has been paid, perhaps because it has been generally regarded as a fundamental property of the whole pneumococcus species. Unfortunately, however, the criteria and technique employed in determining this property have varied, and in particular the differentiation of bile resistance from bile insolubility does not seem to have been clearly defined. However, in spite of the limitations of definition it should be recalled that not only have intermediate pneumococci which are insoluble in bile been described by Kimura, Sukneff and Meyer (1928), but that even S pneumococci which are more or less insoluble have been described by Paul (1927b), and by Whittle (1928). With R pneumococci, bile resistance has been observed, by Reimann (1927), Neufeld and Levinthal (1928) and Neufeld and Etinger-Tulczynska (1930); that is, it takes longer for lysis to take place and this lysis may be incomplete. Occasionally, bile-insoluble R forms have been reported, such as one of the six strains tested by Downie, Stent and White (1931). Furthermore, in the preceding article reference has been made to the fact that in experiments upon the dissociation of S pneumococci in media containing bile, many R forms appeared which proved to be partially bile soluble. There were also a few instances in which forms, resembling true Rs, proved to be completely bile insoluble on their first few transfers. This finding was, however, rather unusual.

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[1] The type of serum bouillon is not specified but in the interpretation of this finding it is important to recall that not only does human serum, but sera from certain animals such as the horse, guinea pig, etc., occasionally contain agglutinins for R pneumococci in low dilutions. In swine serum, Kelley (1932) has demonstrated them in a dilution as high as 1:640.

It also seems pertinent to mention another (unpublished) observation of this type, namely, that of a II-R strain which, after prolonged cultivation on laboratory media, became insoluble in bile. This finding was demonstrated by a series of consecutive tests performed with the same technique. Two strains (I-R and II-R) were obtained by the author in 1926, through the kindness of Dr. O. T. Avery of the Rockefeller Institute for Medical Research. During the ensuing seven years they were kept upon rabbit-blood agar in the ice-box with transfers every two to three weeks. When received both strains were completely soluble in bile, but in testing this property six months later it was noted that the II-R strain had become somewhat resistant to bile. When tested a year later it was bile insoluble, whereas the I-R strain was only partially insoluble. These properties have apparently remained fixed during the subsequent five years. Other properties of these strains studied over this period, have shown little change except that of autolysis in saline solution. Originally, a suspension of organisms of each strain underwent autolysis in saline solution within eighteen hours, but a year later this property was also permanently lost. Both strains were avirulent when received and efforts to revert them, either by in vitro (first tried in 1927), or in vivo (first tried in 1928) methods have been unsuccessful. Our bile-insoluble strain has retained its original colony appearance, and its ability to grow diffusely in broth. It is, nevertheless, now practically indistinguishable from a *Streptococcus viridans* and has been so for five years. A somewhat similar observation has been recently noted with another II-R strain which had been kept in the laboratory for 11 months. After nine months this strain lost its ability to be reverted by Griffith's method, its colonies became rougher, and it became almost but not wholly insoluble in bile. These strains have been described because they exemplify the fact that bile solubility may not be a fundamental property of R pneumococci, but rather one which may be lost like many of the other features which usually differentiate R from S pneumococci.

That there may be various degrees of roughness or stability of the R forms as expressed in terms of the ease or difficulty with which spontaneous or induced reversion to the parent S forms takes place, has been intimated by Griffith (1928), Neufeld and Levinthal (1928) and by Sia and Dawson (1931). To our knowledge, however, there has been no concerted attempt to codify or classify the group on this or other bases, although the observations of Neufeld and Levinthal (1928) are important in this respect. These authors employed the reversion method of Griffith (1928) on three R strains. With the first strain the bile-solubility reaction was rapid and complete; with a second strain this reaction was prolonged but complete, and with a third it was incomplete. Successful reversion was accomplished only with the first strain.

It now seems evident that R pneumococci may also undergo further "degradative" dissociation. An instance of this appears in the previous article in which a bile-insoluble form giving rise to very rough, large colonies was produced by the prolonged cultivation of a II-*b* strain in media containing 75 per cent bile. Another R dissociant has been recently described by Dawson

(1933a). This form was produced by aging R colonies on blood agar for six to seven days at 37°C. By this method daughter colonies were produced which, when picked and transferred, gave rise to colonies with an extremely rough and irregular surface, and these in turn on aging developed a spreading filamentous type of marginal growth. The morphology of the organisms from these colonies consisted of plump cocci and coccobacilli in long chains together with large numbers of granular elements. In liquid media there was a sedimented type of growth resembling a tuft of cotton in the bottom of the tube. These organisms possessed relatively little virulence for mice. They were bile soluble. It may be pointed out, however, that this variant approaches in its colony type and its growth in broth, the streptococcus-like forms supposedly derived from pneumococci, to which reference in the literature has been made.

On the basis of this finding Dawson (1933b) has suggested that it may be necessary to revise the terminology of pneumococcus variants. He believes that the new form he has described may represent the "true" R form of the pneumococcus species, and that the form previously regarded as an R might be better termed an S, and the S form in turn as an M (mucoid). Although we have not as yet employed this terminology it has also seemed to us (as will be subsequently shown in this paper) that the use of some new symbol or symbols to designate the "very rough" forms is necessary.

GENERAL METHODS

Growth characteristics. As stated in the preceding article cultures were grown upon fresh rabbit-blood agar plates and colonies were studied under the microscope after thirty-six to forty-eight hours.

The character of the growth in broth (diffuse or granular) was determined from a twenty-four-hour culture in rabbit-blood broth, that is, in tubes containing 5 cc. of meat or yeast extract broth to which 2 drops of fresh rabbit blood had been added just prior to the planting of the culture. It is our belief that the concentration of rabbit serum in the medium is so small as to render negligible the chance of agglutination of the strain to be tested by the possible presence of anti-R agglutinins in rabbit sera.

Agglutination reactions. The agglutination methods employed for the differentiation of R and intermediate pneumococci have been described in the preceding article.

Reversion methods. Besides the use of Dawson's in vitro method reversion tests were also conducted by the in vivo method of Griffith (1928). I-S and II-S strains, grown in 50 cc. of yeast extract broth, were centrifuged and taken up in one-tenth their original volume of saline. This material was placed in sealed tubes which were immersed in the water bath at 56°C. for twenty minutes. It was subsequently tested for sterility. Mice were inoculated subcutaneously with 0.5 cc. of an eighteen-hour broth culture of the R form to be tested, and 0.5 cc. of the homologous heat killed vaccine. A control mouse which received 0.5 cc. of the heat killed vaccine alone was also included in each reversion experiment. If 10 mice are injected with equal amounts of an easily revertible II-R culture together with killed S substance, it has been our usual experience that in but six or at best eight will reversion take place. For this reason tests of this type were run in duplicate.

Bile solubility. Although this test is widely used, various techniques seem to have been employed by different investigators for determining the lytic action of bile on cultures of pneumococci grown in fluid media. Methods of expression of the results have also varied. Furthermore, in spite of known variables such as that exerted by the hydrogen-ion concentration of the suspension of organism to be tested, it has seemed probable to us that unknown variables also exist. In our hands at least, occasional control tests have failed to give clear-cut reactions. For this reason we have not attempted to quantitate our results but have described them in three terms, namely: complete solubility; partial solubility; and insolubility.

Strains to be tested were grown for eight to sixteen hours in 5 cc. of meat or yeast extract broth (pH 7.2) to which two drops of 10 per cent glucose solution were often added to promote rapid growth. The organisms were centrifuged and re-suspended in 1 cc. of saline solution. To 0.5 cc. of this suspension 0.5 cc. of ox bile was added, and the mixture well shaken and incubated in the water bath at 37° for two hours. The other 0.5 cc. portion of each suspension to which 0.5 cc. of saline had been added, was used as a control. Strains of known bile solubility and insolubility were set up as controls with each series of tests and needless to say, if the reactions were not clear-cut in these controls, the results were discarded. As a substitute for ox bile, solutions of sodium taurocholate or sodium desoxycholate were occasionally used, but in our hands ox bile has proved more satisfactory. It is well known, however, that different samples of ox bile may vary in activity and for this reason only those samples were used in which 0.1 cc. of ox bile would completely dissolve 0.9 cc. of a suspension of S pneumococci.

Nomenclature. Symbols employed for expressing the results of these experiments have been devised for purposes of personal convenience and not necessarily for permanent application. The variety of variants (or various phases) arising in the dissociative patterns which have been found have been classified only according to easily demonstrable characteristics on which our terms have been based, namely: the colony on rabbit-blood agar, and the character of growth in rabbit-blood broth. Using the typical R colony (see fig. 1) as a standard, the terms R′ and R″ have been used to designate colonies with increasingly rough surfaces. The shape of the R′ colony is generally that of an R, its size is often larger; its surface is often studded with tiny irregularities or excrescences (see fig. 2). The R″ colony (see fig. 3) varies in size from that of an R to an S and its shape and outline may be very irregular. It may be flat with a sharp or filamentous edge or it may be cone-shaped with a heaped up, irregular surface suggesting an aerial view of a rugged mountain. Both R′ and R″ colonies give rise to a surrounding green or faintly hemolytic zone. The morphology of most of the organisms from R′ and R″ colonies resembles that of the R pneumococci. Bacillary and irregular forms and even granular elements may be present.

Most of the organisms from R and R′ colonies which we have studied have been completely or partially soluble in bile and most of these give an R type of agglutination with the thread test. Most of those from the R″ colonies on the other hand, have been partially or totally insoluble in bile, and grow granularly in broth so that the thread-test reaction cannot be satisfactorily employed for the detection of species specific agglutinins. Occasionally R′ strains have been reverted to S forms. None of our R″ strains in this particular series of experiments have been reverted. The terms RG, R′G and R″G have been applied respectively to the organisms from R, R′, and R″ colonies which, on the first few transfers in rabbit-blood broth give rise to a granular growth. It is possible that the use of the term RG may be confusing in that the letter G has been employed by some to represent gonidial forms, as by Hadley (1933). On the other hand, our adoption of this symbol is based on the earlier usage of DeKruif (1921 and 1922) of the symbol G (granular) as opposed to D (diffuse) in his studies on variants of *Bacillus lepisepticus*. With the pneumococcus forms the G property may be lost on repeated transfers in fluid media.[2] Different types of granularity may appear in the broth cultures of RG forms varying from that of an even suspension of tiny particles, which in the course of forty-eight hours will sediment to the bottom of the tube, to the presence of large whitish flakes. We have seldom noticed the type of growth exhibited by Dawson's variant (1933a) in which the growth simulated a tuft of cotton in the bottom of the tube, a type of growth often seen in *Streptococcus viridans*-like organisms isolated from the throat of human beings. As already intimated, most of our RG strains were either partially or totally insoluble in bile. Those in which insolubility was a persistent characteristic have been designated as RG (streptococcus-like) forms.

EXPERIMENTAL

Dissociation of S, intermediate and R strains by in vitro methods. A variety of methods for the dissociation of S pneumococci were tried during the course of these experiments which will not be reported in detail. These included a series of experiments in which S, intermediate and R forms were transferred daily in broth containing various dilutions of yeast-absorbed optochin, according to the technique of Morgenroth et al. (1925). The results were irregular. With most of the strains we got little or no detectable evidence of dissociation; with others we got a dissociative pattern similar to that reported with bile in the preceding publication, in which S strains became more or less stabilized as intermediates, recalling results obtained by Klumpen (1932). In still other optochin experiments a few forms were derived which produced unusually rough (R′ and R″) colonies. Examples of some of the colonies produced under these conditions appear in figures 4 and 5. Some of these strains grew granularly on the first few transfers in rabbit blood broth. Others grew diffusely and gave an R type of thread test agglutination. Still others which grew diffusely, showed little ability to be agglutinated by homologous or heterologous anti-pneumococcus serum. All of these forms were avirulent and all were either bile soluble or partially bile soluble. Attempts to revert these strains by either the method of Dawson or Griffith were unsuccessful. No systematic study of these "irregular" forms was done because it has been felt that methods are not yet available for their consistent production.

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[2] It is recognized that different strains of *Streptococcus viridans* exhibit different types of growth in broth and according to Topley and Wilson (1932) those growing in short chains produce a turbidity which is almost uniform; others growing in longer chains give rise to a granular growth forming a powdery deposit at the bottom of the tube or on its sides, similar to the growth produced by *Streptococcus hemolyticus*. These characters are, however, far from being fixed in each individual strain and in this laboratory several strains (isolated from the blood of patients suffering from bacterial endocarditis) have given rise to a granular growth when first isolated, but when carried for a few transfers in broth this has changed to a diffuse turbidity.

Another method applicable to some of the more hardy intermediate and R strains is that of allowing a culture of the strain to be tested, to grow at room temperature on a blood agar plate. This procedure is somewhat similar to that employed by Dawson (1933a) in producing his variant which according to our terminology might best be termed an R″. It is recognized that colonies of S pneumococci generally attain maximum size within forty-eight hours, subsequently undergo some autolysis with or without the formation of daughter colonies, and die within four to eight days if left in the incubator or at room temperature.[3] With the intermediate and R forms obtained as a result of growth in bile or optochin we found that colonies would often continue to increase in size, either as a whole or by the formation of peripheral daughter colonies, over a period lasting from twenty to thirty days depending somewhat upon the particular lot of medium employed. By sealing the plate with adhesive tape, growth could be extended over these long periods. Frequently we observed the dissociation of *-c* and *-d* forms to Rs as a result of this process. An example of a dissociating colony of this type is shown in figure 5. Graphs representing serial measurements of two colonies, one a II-S strain and the other its II-*d* dissociant, both of which were grown on the same plate, appear in text-figure 1. On a single occasion a bile soluble R′ culture, produced as a result of growth in broth containing yeast absorbed optochin and allowed to grow at room temperature for 16 days, gave rise to daughter colonies, which when picked and transferred produced rough (R″) colonies; the latter organisms grew granularly in broth and were bile insoluble, or were, in other words, what we have termed R″G streptococcus-like forms.

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[3] The ability for an occasional strain of S pneumococci to grow at room temperature has been recorded and the author has had the opportunity to study such a strain which has been described by Rakieten (1930).

In vivo dissociation of R forms. This method has been employed by Reimann (1927b), who has shown that R forms appear in cultures of S pneumococci growing in agar foci subcutaneously embedded in guinea pigs, and in agar enclosed in vials subcutaneously embedded in rabbits. He noted that pure cultures of R forms remained viable in subcutaneous foci for weeks. Later (1929) work by this author showed that R organisms inoculated into agar foci in mice would show certain colony alterations suggestive of the intermediate forms.

Our experiments of this type consisted of the introduction of a culture of R organisms together with an irritant or toxic substance into or beneath the skin of rabbits, guinea pigs, and mice, of aspirating material from the resulting abscess or inflammatory area, and of studying the bacterial forms isolated from day to day.

Methods. A II-R strain was used in all recorded experiments. It represented the twelfth transfer of a II-S grown in type II anti-pneumococcus serum broth and could be readily reverted to its parent S type by Griffith's method but was of sufficient stability so that it did not revert spontaneously when injected into mice alone. Eight mice survived the inoculation of 0.5 cc. of a sixteen-hour culture. Other characteristics of this strain were also quite typical in that it was moderately bile soluble, grew diffusely in broth, and gave a typical thread-test agglutination reaction. When 0.3 to 0.5 cc. of a broth culture was injected intra- or subcutaneously into rabbits or mice, either no lesion was apparent on the following day, or there was a small (2 to 3 mm. in diameter) erythematous area which disappeared within forty-eight hours. We were never successful in recovering R pneumococci or other variants from such areas on the day after inoculation or on subsequent days.

The use of an irritant or toxic substance simultaneously or previously injected intra- or subcutaneously with the II-R culture would give rise to a more satisfactory dermal lesion of varying size (5 to 20 mm.) depending on the dosage or material used. In rabbits this lesion would remain red and indurated for two to five days and would then form a small, pale, subcutaneous abscess which would generally heal within five to ten days without a fistula. In mice the same general picture was encountered although the abscess was apt to persist for a longer period if it did not rupture through the skin.

A variety of irritant substances were tried including heat-killed S pneumococci, 95 per cent alcohol, dilutions of carbolic acid, and xylol. Of these the most satisfactory proved to be a 20 per cent solution of xylol in mineral oil (a 50 per cent solution was used with mice). This was injected in 0.1 to 0.3 cc. amounts eight to twenty-four hours prior to the injection of an equal amount of an eighteen-hour II-R broth culture. We have also used heated sputum for this purpose.[4] A large sample (200 cc.) of sputum, obtained from a patient suffering from a chronic non-tuberculous, pulmonary infection, was diluted with one part of meat infusion broth, autoclaved, tested for sterility and kept in the ice-box for several months. This material was moderately toxic for mice, for when injected intraperitoneally in 0.5 cc. quantities, about half the animals died. The intradermal dose for rabbits and guinea pigs was 0.2 to 0.3 cc. (the subcutaneous dose for mice was 0.1 cc.) which, when injected simultaneously with an equal amount of the II-R culture, gave rise to a lesion similar to those caused by xylol. Occasionally rabbits were encountered which failed to react with heated sputum on the first inoculation but when the material was re-inoculated ten days later the lesion above described would occur. In performing the inoculations, the animal to be injected was shaved over its back and the skin was moistened with alcohol. In rabbits three to six intradermal injections were made including a heated sputum or xylol control, which was injected alone. With guinea pigs two to four intradermal injections were made, and with mice one subcutaneous injection. Separate mice were used as controls. Aspiration of the skin lesions was done on subsequent days by moistening the overlying skin with alcohol and injecting 0.1 cc. of sterile salt solution into the lesion, and then with the same needle in place, withdrawing as much fluid as possible. Material thus obtained was spread upon a blood agar plate and incubated for thirty-six to forty-eight hours. Differential colony counts of the resulting growths were made. Representative colonies were picked and of the strains thus isolated, the character of their growth in broth, their thread-test agglutination and bile solubility-reactions were studied. In many instances (see under Results) the revertibility of these strains to S forms was also studied.

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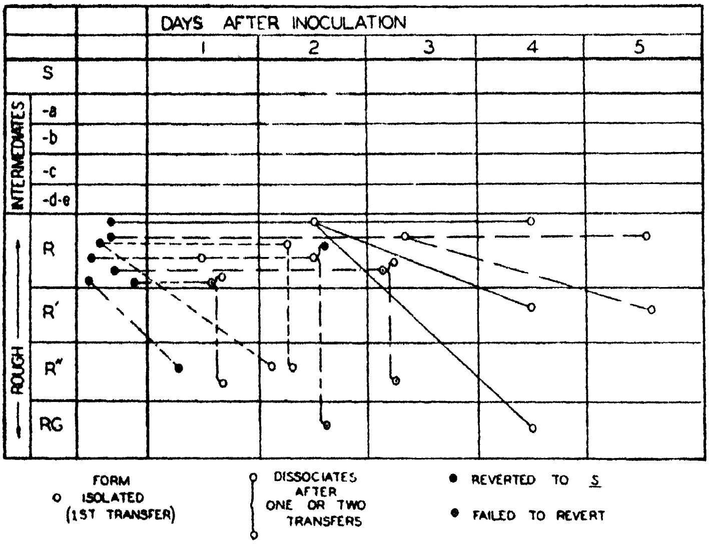
[4] The use of sputum was primarily employed because it seemed conceivable that mucinous substances present in sputum might aid in bringing about an R → S reversion. However, no evidence that such was the case was ever noted.

The possibility of obtaining contaminants or bacteria derived not from the strains inoculated, but from the rabbit is appreciable. A few colonies of staphylococci were frequently seen upon the plated cultures from both the lesions in which II-R forms had been injected and from the control lesions. Our belief that the organisms recorded in our results (text-figs. 2 and 3) were in all probability not contaminants has, however, been based upon two facts: (*a*) that no organisms which gave rise to rough colonies surrounded by a greenish or faintly hemolytic zone were encountered in the control lesions; and (*b*) that successful reversion to the type of its parent S strain was occasionally accomplished with these suspected dissociant forms isolated from the dermal lesions.

Results. Seventeen dermal lesions of the type described were produced and studied in rabbits, fifteen such lesions were studied in mice, and eight in guinea pigs. Primarily it was found that in all three of these experimental animals, dissociation of the injected II-R strain could be frequently demonstrated. This dissociation consisted either in a reversion to the parent S strain (which only occurred in mice) or a degradation to R′, R″, and RG forms. In rabbits the latter phenomena occurred in about one-third of the lesions studied and the very rough forms isolated were more or less similar to those noted on one occasion in the in vitro bile experiments recorded in the preceding paper, and on a few occasions in the in vitro optochin and aging experiments reported in this paper.

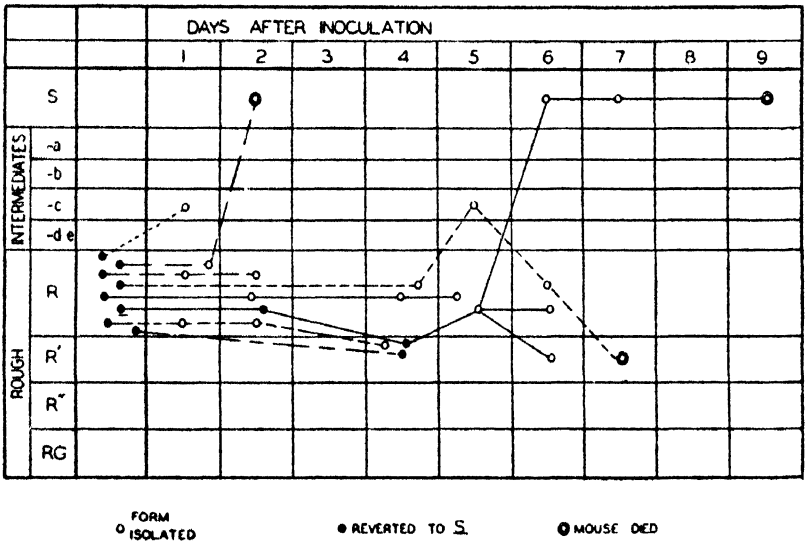
The results of cultures from seven different dermal lesions in the rabbit, appear in text-figure 2 and show the period in which R forms survived and the days on which R′ or R″ colonies were detected. It was a usual experience in rabbits that on the first two days following inoculation, II-R pneumococci could be recovered in moderate numbers from the majority of the lesions. The properties of these strains were as a rule similar to those of the inoculated strain, except that it was more difficult to revert them to their homologous S form. On the third to the fifth day after inoculation cultures from the dermal lesions were either negative for R forms or they were present in small numbers resulting in about two to ten colonies. It was during this period (when the growths from the lesions were scanty) that R′ or R″ colonies (see fig. 7) would appear in small numbers. Generally their appearance would herald the fact that cultures on subsequent days would be sterile. Not infrequently incidents occurred which suggested that the dissociative process initiated within the lesion would continue in vitro, or, in other words, that the "stability" of the II-R strain had apparently been altered by its habitation within the lesion. For instance from four of the lesions listed in text-figure 2, R″ of RG forms were not detected in the plated culture made directly from the lesion, but appeared



TEXT-FIG. 2. VARIANTS ISOLATED FROM SEVEN DERMAL LESIONS IN THE RABBIT subsequently in cultures from a single R colony which had been picked and transferred once in blood broth. An observation of this type may be recorded as follows: one of the animals was used twice, the second experiment being performed fifteen days after the first. R forms disappeared more rapidly from the lesion than on the first occasion and on a plated culture made three days after the second inoculation, a single, fairly typical R colony was found, together with a few colonies of *Staphylococcus albus*. This single R colony was picked, inoculated into broth and replated. The second plating revealed a mixture of R and R″ colonies (see fig. 8); organisms isolated from the R colonies grew diffusely in broth and were moderately bile soluble, whereas those isolated from the R″ colonies produced a granular growth in broth and were bile insoluble. The attempt was made to revert both of these strains. The R strain was successfully reverted to a II-S by the Griffith method; the R″G strain did not revert by this method. Observations of this type were not conducted on single cell cultures.

With guinea pigs the survival time of R forms within the dermal lesions was shorter than with rabbits in that cultures made after twenty-four hours were nearly always sterile. On the other hand with mice, although the experiments were often terminated by the fact that the lesion broke down following its first or second puncture with the aspirating needle, II-R pneumococci or their dissociants were occasionally recovered up to six days after inoculation.

The in vivo method in these three animals produced R′, R″ and RG forms with more consistency than any of the other methods we have employed, in that thirteen variants which gave rise to R′ colonies, and twelve which gave rise to R″ colonies were observed in the course of the experiments. Many of these forms grew poorly or died out in the types of media usually employed for the culture of S pneumococci and the strains were lost. Others maintained their original characteristics through many transfers in rabbit serum broth, although (as mentioned under Methods) the G character, or the ability of an R′ or R″ to grow granularly in broth, was often lost. The revertibility of these strains seemed to decrease somewhat in proportion to the roughness presented by the colony surface. We never succeeded in reverting an R′ or R″ form isolated in these experiments, back to its parent strain by the in vitro method of transfers in rabbit-serum broth. On the other hand of 11 R strains (isolated from the first to the fourth day after inoculation) 6 were reverted by Griffith's method; of 5 R′ strains similarly isolated 3 were reverted; and of 5 R″ or RG strains, none was reverted directly, although with one of the R″ strains (isolated from a single colony from a twenty-four-hour guinea pig lesion) an R form appeared in the first blood-broth transfer and this was subsequently reverted to a II-S.



Text-Fig. 3. Variants Isolated from Eight Subcutaneous Lesions in the Mouse

No attempt has been made to study the properties of these variant strains in detail; emphasis has rather been laid upon the fact that dissociation of a II-R strain could be induced in this particular environment. In addition to this fact the differences in the dissociative patterns obtained with the different animals employed was another striking feature of these experiments. Such differences may be seen at a glance by comparing the dissociative pattern shown in text-figure 2, representing results obtained in the rabbit, with that in text-figure 3, representing results with mice. In the rabbit the trend of dissociation was essentially "degradative." In mice, on the other hand, R forms occasionally survived for longer periods of time, showed less tendency to dissociate into the very rough forms and would occasionally revert directly to S forms with the resulting death of the inoculated animal. Cultures from 8 sample lesions in mice are shown in text-figure 3. Here it will be seen that two of the mice died as a result of the "spontaneous" reversion of the injected II-R strain. This occurred without the demonstrable production of intermediate forms. In two other mice intermediate II-*c* forms were detected but they did go on to further reversion, recalling the observation made by Reimann (1929) in an earlier experiment of this type.

The results in the guinea pig experiments have not been shown graphically. They resembled those obtained in the rabbit, although the survival time of the inoculated R forms and their dissociants was shorter. One difference noted in the guinea pig experiments was that still another presumably variant form, not seen in the rabbit or mouse experiments, was derived from the dermal lesions in this animal. This form gave rise to colonies which did not produce a prominent peripheral zone of "greening" or hemolysis of the underlying medium. Attempts to revert these latter forms to S pneumococci were unsuccessful.

DISCUSSION

In the experiments just described several pneumococcus variants originally derived from S and R forms have been occasionally observed which do not seem to belong to the well recognized intermediate and R forms of the pneumococcus species, but appear to represent dissociants of the latter forms, particularly the Rs. This is an observation somewhat similar to that which Dawson (1933a) has recently made. The criterion upon which our definition of these atypical variants has been partially based is that the suspected organism should be reverted to its parent S type, and upon this procedure rests the claim that the atypical R forms described were actually pneumococci. We only succeeded in fulfilling this postulate in a few instances but enough of the atypical R forms were reverted to S forms to show that "atypical" R pneumococci do exist. In general, the colonies of the atypical variants described are rougher than those produced by R forms, and as roughness increases the strains become more refractory to reversion and more resistant to the lytic action of bile. Some were bile insoluble, and some gave rise to a granular growth in broth similar to organisms of the *Streptococcus viridans* group. In this respect the findings support the earlier observations, though not necessarily the conclusions, of Rosenow (1910 and 1914); of Morgenroth et al. (1925), and others who believed in the transformation of pneumococci into "streptococci" and vice versa. Our atypical variants might be broadly considered as R pneumococci if the application of the term R be applied to cover a large and heterogeneous group, but for practical working purposes we have applied the symbols R′, R″ and RG rough granular (streptococcus-like) to designate certain members or variants of the R group. This is not necessarily an adequate or final method of designating these strains, for some of the properties on which the terminology has been based are occasionally unstable. However, it has not been our major aim in this paper to describe in detail all of the different variant forms which have been isolated in the course of these experiments and to individualize them by attaching an identification tag to each, but rather to confirm the view that so-called degradative dissociation of R pneumococci does take place under certain environmental conditions, thus giving us a wider conception of the potentialities for variant production by the pneumococcus. Armed with these facts one may approach the study of pneumococcus-variant production in human beings with the realization that, if dissociation of S pneumococci does occur in human infections or carrier states, the variant forms produced are not *necessarily* going to be identical with those which may be produced *in vitro* or even in experimental infections in laboratory animals.

SUMMARY

1. Certain definitive features of R pneumococci have been discussed and attention has been called to the fact that classical R pneumococci have usually been derived by growth of S forms in homologous anti-sera, whereas so-called atypical R pneumococci have generally been produced in other environments.
2. Methods of producing dissociants from R pneumococci have been described and, for practical purposes alone, the attempt has been made to subdivide the group.
3. Some of these R dissociants which were bile insoluble and which produced a granular growth in broth have been described. They appear to be indistinguishable from certain strains of *Streptococcus viridans*.

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PLATE 1

All of the colonies shown in figures 1 to 9 are forty-eight hours old unless otherwise specified.

Fig. 1. I-R colonies (× 20). Note the typical round, slightly irregular dome shape. The outline is a little distorted due to a crowding effect.

Fig. 2. II-R′ colony (× 40).

Fig. 3. R″ colony (× 40). Irregularity of surface outline and shape is the characteristic feature.

Fig. 4. R′ colonies derived from the third transfer of a group IV-S strain in media containing yeast and optochin (× 20).

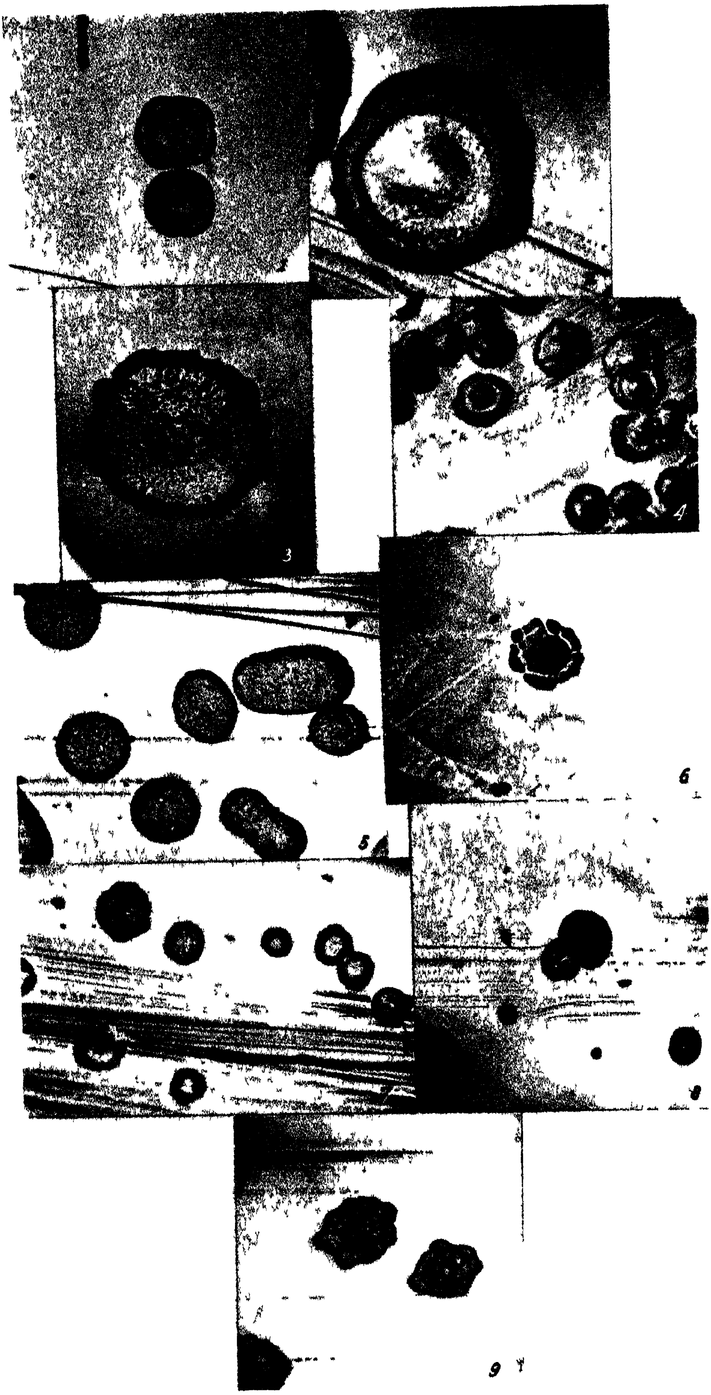
Fig. 5. R″ colonies (× 24) from a later transfer of the strain shown in figure 4.

Fig. 6. The development of rough (R) daughter colonies from the periphery of a II-*d* colony (× 20). The forty-eight hour II-*d* colony had been allowed to stand at room temperature for three days.

Fig. 7. R′ and R″ colonies (× 20) colonies isolated from a dermal lesion in a rabbit previously inoculated with a II-R.

Fig. 8. Contiguous R and R″ colonies (× 20) representing strains derived from a dermal lesion in the rabbit. The R″ variant appeared in a culture (picked from a single R colony) which had been transferred once in broth. The R strain was successfully reverted to its parent II-S type.

Fig. 9. R″ colonies (× 20) derived from a dermal lesion in a guinea pig inoculated with a II-R.



(John R Paul Pneumococcus variants)

THE SYNTHESIS OF AROMATIC AMINO ACIDS FROM INORGANIC NITROGEN BY MOLDS AND THE VALUE OF MOLD PROTEINS IN DIETS

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Although there has accumulated a certain amount of information on the chemistry of microbial proteins, very little is known as to the identity of specific amino acids found in such proteins synthesized from inorganic nitrogen or simple organic nitrogen compounds. Indeed, it is not always acknowledged that bacteria have this synthetic power. Only nine years ago, A. I. Kendall (1925) stated "In this respect *plant cells differ from bacterial* and animal *cells in that they* utilize, through the chlorophyll of their leaves, solar energy and *can synthesize protein from mineralized nitrogen.*" (Italics not in original.) Unless one can consider bacterial protoplasm as being free of proteins, this concept of Kendall's cannot be entertained. The fact that many species of bacteria make a heavy growth in media whose only source of nitrogen is in the form of nitrates, ammoniacal salts, or elemental nitrogen is too well known to need elaboration. Vorbrodt (1919, 1921, 1926) and Heck (1929) have furnished splendid summaries of the work done and have themselves clearly shown the conditions regulating protein synthesis by molds.

Much of the work on the amino acids in microbial proteins can be discounted or ignored from the point of view of synthesis of these amino acids, since the organisms were grown on peptone, beef, malt wort, or other amino acid-containing substrate, and the specific amino acids of the substrate may well have been used as "bricks," using Fischer's figure of speech, in building a new protein structure, the microbial protoplasm. Only in case the microbial cell contains amino acids formed from inorganic nitrogen or from an organic source simpler than the amino acid can we properly speak of *synthesis*. Therefore, only those papers which may possibly deal with actual synthesis of amino acids will be reviewed here.

Tamura in 1913 and 1914 published four papers on the proteins of bacteria. Most of the work was done with a substrate containing beef, peptone, etc., but in the second paper (1913), *Mycobacterium lacticola* is stated to have been grown on a medium consisting of K_2HPO_4, $MgSO_4$, asparagin, ammonium lactate, glycerol, and water. From organisms grown on this substrate was extracted material giving the following analysis:

Arginine	10.92
Histidine	1.11
Lysine	1.18
Ammonia	0.54
Phenylalanine	5.14
Proline	5.76
Valine	1 57
Other amino acids	51.12
N in unknown form	22.66
Tryptophane	Present

Popper and Warkany (1925) grew the tubercle bacillus on a medium whose nitrogen source was asparagin and found that the dried bacilli contained 1.1 per cent tryptophane and 1.4 per cent tyrosine. These authors properly pointed out that some bacteria, at least, resembled plants rather than animals in their ability to synthesize proteins.

Logie (1920) grew *Bacterium coli* for several generations on synthetic media containing ammonium lactate, sodium asparaginate, glycerine, salts and water, and found that after adding an alkali and precipitating the cells, a strong Hopkins-Cole test for tryptophane was obtained. Much less definite evidence of tryptophane synthesis by other bacteria was discussed by Logie.

No rigid demonstration of specific amino acid synthesis by yeast was found, due possibly to the persistent belief that "bios" is necessary for the growth of all yeasts. The recent work of Fulmer, Nelson and White (1923), and Werkman (1925), showing that some yeasts, at least, grow readily in a purely synthetic medium, may stimulate work along this line. The work of Thomas (1921) and associates, during the years 1913–1921, had to do with commercial yeast, produced ordinarily in those years from beer wort, and molasses. Tryptophane and tyrosine were shown to be present by definite tests, and by isolation in crystalline form, but can hardly be said to be synthesized.

In the study of amino acid synthesis by molds, *Aspergillus niger* has been used. Abderhalden and Rona (1905) grew this organism on a medium containing $MgSO_4$, KH_2PO_4, KCl, $FeSO_4$, glucose and either KNO_3, glycine, or glutamic acid. Irrespective of the sources of nitrogen, about the same amounts of glycine, alanine, leucine, glutamic acid and aspartic acid were found in the mold growth. No tyrosine, phenylalanine or proline was found. Apparently tryptophane was not tested for. Thomas and Moran (1914) studied this same species, but they do not state the composition of their medium, merely referring to an earlier paper for details of technique. In this paper (on yeasts) they state that their yeast was obtained from a commercial source. Thomas and Moran obtained positive xanthoproteic and glyoxalic tests showing the presence of tryptophane. Cystine, incidentally, was absent. Due to the lack of details of technique, one cannot but assume that these authors did not demonstrate *synthesis* of any particular amino acid.

Since Abderhalden and Rona's well known work, Vorbrodt's is by far the most clear-cut on the subject. In one (1919) of a series of papers, he reports growing *Aspergillus niger* on a synthetic medium whose nitrogen source was ammonium nitrate, and isolated tyrosine, leucine and alanine definitely and possibly identified phenylalanine and proline. Since Vorbrodt's finding that *Aspergillus niger* synthesized tyrosine is not in accord with Abderhalden and Rona's data on the same species, it opens a new field for investigation. To be sure, Abderhalden and Rona used KNO_3 and Vorbrodt, NH_4NO_3. The recent work of Raistraick and associates (1931) has clearly demonstrated the synthesis of several cyclic non-amino compounds by many species of molds.

Recapitulating the above review, it has been found that *Myco-*

bacterium tuberculosis may synthesize tyrosine and tryptophane from asparagine and *M. lacticola*, phenylalanine and tryptophane from asparagine and ammonium lactate, that *Bacterium coli* may synthesize tryptophane from ammonium lactate and sodium asparaginate, and that the ability of *Aspergillus niger* to synthesize tyrosine is not agreed upon. Abderhalden and Rona failed to find it synthesized from KNO_3 and Vorbrodt definitely isolated it from the mycelium grown in media containing NH_4NO_3.

The problem has a possible practical application. Robertson (1920) would supply the population of the future with proteins from microörganisms which would be grown on cheap carbohydrate material, inorganic salts and inorganic nitrogen. But apparently neither Robertson nor Pringsheim and Lichtenstein (1920) who, due to war exigencies, fed animals on straw treated with nitrogenous material in which fungi had grown profusely, appreciated the limitations of such foods, if Abderhalden and Rona's findings, so often cited in the literature, were the complete picture. One might also call attention to the common conception (Mendel, 1923) that certain herbivorous animals may obtain some of their needed amino acids from those built up by the bacteria growing in the gut. But to be fair one must also point out this is far from being universally accepted, due to lack of clear-cut evidence (Mitchell and Hamilton, 1929). It is to reopen the whole subject of synthesis of aromatic amino acids by microörganisms that this present work is reported.

EXPERIMENTAL

A medium of the following composition was used throughout.

$Ca(NO_3)_2 \cdot 4H_2O$	2 grams
KH_2PO_4	2 grams
$MgSO_4 \cdot 7H_2O$	1 gram
$(NH_4)_2SO_4$	2 grams
$FeCl_3$	Trace
H_2O	1000 cc.
Glucose	20 grams

All chemicals were C.P. grade. The medium was placed in carefully washed 1-liter medicine bottles, in 150 cc. amounts, and sterilized for twenty minutes at 15 pounds pressure. The bottles were inoculated by adding 1 cc. of a suspension of spores and mycelium of molds grown on the same medium. After growth in a prone position at room temperature in total or partial darkness, the bottles and contents were heated to 100° in an autoclave, and the contents were filtered. As much of the liquid as possible was pressed out. The combined mycelium and spores were then dried and ground. This material was used for chemical tests or feeding.

The following organisms were used: *Aspergillus niger* (isolated from air, identified by author); *Trichoderma königi* (obtained from Waksman, isolated from soil); *Zygorrhinchus moelleri* (obtained from Waksman, isolated from soil); *Penicillium* sp. (pink, no. 24, isolated from soil by Waksman); *Aspergillus oryzae* (identified by A. T. Henrici); *Aspergillus terreus* (isolated by Lucille Bishop from aborting cow, diagnosis checked by Henrici); and *Penicillium flavo-glaucum* (isolated from air, diagnosed tentatively by Thom).

All the dried cultures gave strong xanthoproteic and Millon tests, the latter suggesting the synthesis of tyrosine. To test for tryptophane, the paradimethylaminobenzaldehyde test was used. All except Penicillum no. 24 of Waksman's collection gave a definite but not particularly strong color test. This species has a pigment soluble in acid solution which made it difficult to use color tests. Equipment for $Ba(OH)_2$ hydrolysis was not available so no information as to the synthesis of tryptophane by this species has been obtained to date. It was necessary to use very young cultures of *Aspergillus niger* to avoid complications with the colored spores of that species. The molds were twice grown again for a repetition of the above experiments with identical results.

To furnish further proof of the synthesis of tyrosine and tryptophane, the former usually, and the latter, universally, considered to be "essential" for the growth and well being of young rats, feeding experiments with *Penicillium flavo-glaucum* were made. Rations were used as shown in table 1.

Rats, twenty-one days old, were placed in individual cages and fed the rations *ad lib.* Daily, 2 drops of cod liver oil and 100 mgm. of Harris yeast vitamin were given each animal. Weight of animals and food consumed were measured weekly. The graph gives the weekly weights for the first series. The work was repeated, with animals from another colony, with essentially similar results.[1]

TABLE 1

Feeding experiments with Penicillum flavo-glaucum

RATION	A	B	C	D	E	F	G	H	J	K
Crisco	15	15	15	15	15	15	15	15	15	15
Salts (McCollum's no. 185, iodized salt for NaCl)	5	5	5	5	5	5	5	5	5	5
Dextrinized starch	51	22	66	66	66	55	35	42	42	31
Agar-agar			5	5	5	5	5			
P. flavo-glaucum*	29	58						29	29	29
Casein (Harris)			9					9		
Gelatin (Difco)				9					9	
Dry egg albumen (Mallinckrodts)					9					
Maize-gluten†						20	40			20

* 29 grams = 9 grams protein. The NH_3 nitrogen was removed by aspiration in Na_2CO_3 solution before analysis; protein = 6.25 N.

† 20 grams = 9 grams protein, obtained from National Corn Products Co.

It will be seen that the mold is a complete protein, that is, it contains at least traces of the essential amino acids, but not an adequate one on a 9 per cent protein level. Tyrosine, tryptophane, or cystine, at least, are probably partly deficient, since rats on 9 per cent mold protein fortified by 9 per cent gelatin did no better than those fed on mold protein at a 9 per cent level. These three amino acids are lacking in gelatin.

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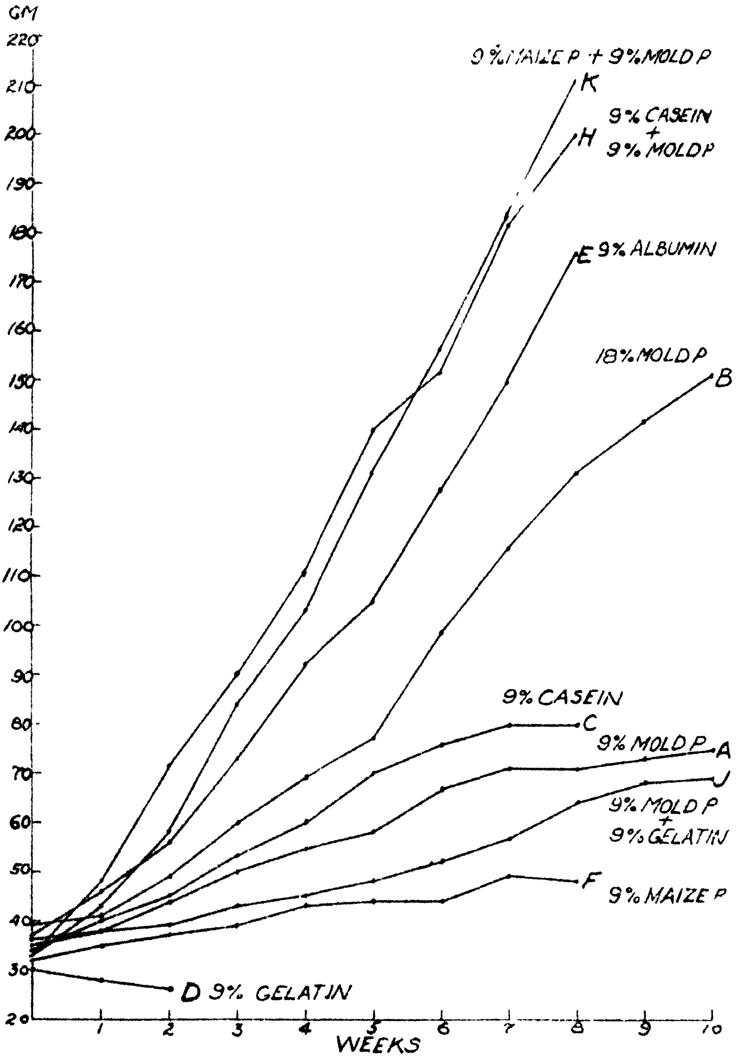
[1] Weekly weights in grams of rats on experimental diets, Series II:

A—9 per cent mold protein. 33, 45, 52, 65, 68, 68, 73, 73, 75, 79
B—18 per cent mold protein. ... 39, 42, 50, 60, 68, 77, 90, 110, 121, 132
C—9 per cent casein.. .... 38, 52, 62, 75, 74, 86, 93, 102, 109, 113
D—9 per cent gelatin...... ..... . 33, 32, 28
E—9 per cent egg albumin. . . 37, 50, 63, 76, 91, 111, 116, 130, 142, 154
F—9 per cent corn gluten protein.. 32, 35, 35, 38, 41, 41, 44, 45, 48, 49
G—18 per cent corn gluten protein.. 33, 39, 45, 53, 61, 64, 73, 90, 98
H—9 per cent mold protein, 9 per cent casein................. 26, 45, 64, 84, 102, 118, 126, 146, 171, 194
J—9 per cent mold protein, 9 per cent gelatin............... 36, 47, 55, 68, 72, 75, 79, 81, 84
K—9 per cent corn gluten protein, 9 per cent mold protein...... 30, 47, 67, 88, 102, 110, 121, 134, 143, 155



WEEKLY WEIGHT OF RATS ON EXPERIMENTAL DIETS, SERIES I

Mold protein (9 per cent) entirely overcame the lysine and tryptophane deficiency known to occur in maize gluten, also fed at a 9 per cent level. Similarly rats fed on a ration containing 9 per cent mold protein and 9 per cent casein, grew normally, showing that the mold had sufficient cystine to overcome the insufficiency of that amino acid, known to occur in 9 per cent casein rations. Rats on a 9 per cent mold protein level did not make rapid progress. In fact the protein at that level is entirely inadequate. At 18 per cent, however, they grew very much better, which leads one to suspect that while all essential amino acids are synthesized, one or more of them is present in very small amounts. Nine per cent protein in a ration is considered sufficient, unless one or more of the amino acids is inadequate. The response of the animals to egg albumin at a 9 per cent level shows that sufficient protein as such was allowed all the animals.[2]

To find a possible limiting amino acid, the paired animal technique favored by Mitchell and Beadles (1930) was used. Litter mates of the same sex and within one gram of the same weight were used in pairs. One animal of each pair was fed ration A (9 per cent *P. flavo-glaucum* protein), and the other received the same ration but with 0.25 gram cystine per 100 grams of ration replacing a like amount of the mold. Each pair received approximately the same amount of food by giving each day to the animal willing to eat the greater amount, the amount the other member of the pair ate the previous day. The greatest care was taken to avoid waste. The animals were weighed every five days. Four pairs of rats were used.

In all four cases the animal receiving the cystine gained more than the other, which results would happen once in sixteen times by chance if the cystine-fortified food were equally as good as the food without cystine. These results in themselves, therefore, are not significant. But 31 separate weighings of pairs were made, and the animal fed on cystine-fortified food in 26 cases gained more per unit of food consumed than its mate. If the cystine were of no value, the "expected" would be 15½. The divergence of 26 from 15½ is 10½. The standard deviation is obtained from the formula $\sigma = \sqrt{.5 \times .5 \times 31} = 2.78$. In case the divergence from the expected is more than twice as much as the standard deviation, the results are to be considered significant. In this case the value is more than 3.5, and hence the results are to be considered significant to a high degree of probability. They indicate that cystine is beneficial in the diet, that is, that cystine is a limiting factor in the make-up of the mold protein when used as a source of protein in diets on a 9 per cent protein level.

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[2] The following growth curve (weight in grams, 5-day intervals) of rats on diet shows that the results were not due to insufficiency of vitamin "B," in 100 mgm. of Harris yeast powder. Each animal was given 2 drops of cod-liver oil and 100 mgm. of Harris preparation daily. A ration was fed *ad lib.* consisting of Harris Vitamin B free casein, 18 grams, starch 62 grams, Crisco 15 grams, salts 5 grams; Rat 1—37, 51, 65, 83, 101, 117, 134, 148, 160, 173, 185. Rat 2—35, 48, 63, 82, 99, 114, 126, 137, 150, 165, 180. Rat 3 (control)—35, 41, 41, 41, 42. Rat 4 (control)—35, 40, 40, 40, 40.

This method of treatment does not take advantage of the magnitude of the greater gain in weight by the cystine fed animals. For this we may use the formulas given by Fisher (1925):

$$\bar{x} = \frac{Sx}{n'}$$

$$\frac{s^2}{n'} = \frac{S(x - \bar{x})^2}{n'(n' - 1)} \quad \text{or} \quad s = \sqrt{\frac{S(x - \bar{x})^2}{n' - 1}}$$

$$t = \frac{\bar{x}\sqrt{n'}}{s}$$

$$n = n' - 1$$

where $x_1, x_2, x_3 \ldots x_{31}$ are the gains per gram of food consumed by the cystine-fed animals minus similar gains of their mates during a five-day period, and n' = the number of weighings of pairs = 31. The calculations of the data show that $t = 5.6$. Using the tables of t of Fisher we find that the highest value given of t for $n = 30$ is 2.750. With this value of t, the probability of the cystine being of advantage would have been 99 in 100. The results here shown, therefore, are much more highly significant than that.[3]

[3] Experiments now (March, 1934) well under way with two additional pairs of rats make results in regard to cystine being deficient still less likely to be fortuitous. Recently a paper by Fildes and Knight (Brit. Jour. Exp. Path. 14, 343-349) demonstrates the synthesis of tryptophane by *Staphylococcus aureus*, *Bacterium typhosum*, *B. aertrycke*, and *Mycobacterium tuberculosis* from inorganic nitrogen or organic non-tryptophane containing substances.

It may be concluded, therefore, that the data on the paired feeding not only confirm the conclusions of the previous work that the proteins of *Penicillium flavo-glaucum* contain all the "essential" amino acids including tryptophane and tyrosine, but also that they demonstrate that cystine, rather than the aromatic amino acids, is present in the protein in such small amounts as to be the first "limiting factor."

In connection with the feeding experiments, it is interesting to compare them with some results of Takata (1929a,b,c) only recently brought to the attention of the author. Takata found that *Aspergillus oryzae* grown in a medium containing dextrine $(NH_4)_2SO_4$ and mineral salts allowed only a slight growth when fed to rats on a 11 per cent protein level. It allowed a greater growth on a 15 per cent level, and on a 50 per cent protein level the animals grew vigorously for two months, then slower. The rate was not affected by the addition of cystine at this time, although the cystine content of the mycelium was low. The tryptophane and tyrosine content of protein extracted from the mycelium was higher than in most proteins. The digestibility of the protein was considered good. It is possible that if Takata had fortified his feed with cystine at the early stages of growth and had controlled the intake by paired animal technique, he would have been able to demonstrate a cystine deficiency. It is also possible that *Aspergillus oryzae* is low in cystine but, that unlike in the case of *Penicillium flavo-glaucum*, some other component was the first limiting factor when used as a source of protein for rats on diet.

SUMMARY

1. *Aspergillus niger*, *A. oryzae*, *A. terreus*, *Trichoderma konigi*, *Zygorrhynchus moelleri*, *Penicillium flavo-glaucum* and an unidentified species of Penicillium, when grown in a synthetic medium whose only *N* source was $Ca(NO_3)_2$ and $(NH_4)_2SO_4$ gave strong Millon tests for tyrosine and all save one gave a positive paradimethylaminobenzaldehyde test for tryptophane.

2. When used as a source of protein at a 9 per cent level in an otherwise complete diet, the dried mycelium of *P. flavo-glaucum*

allowed only slight growth of young rats. On an 18 per cent level the growth was much better.

3. When the dried mycelium was used (9 per cent protein) in rations containing also 9 per cent gelatin no better growth resulted than when the diet containing 9 per cent mold protein alone was fed.

4. When the mycelium was used (9 per cent protein) in rations containing also 9 per cent protein as casein or maize gluten, a normal rapid growth resulted.

5. Paired animal feeding experiments demonstrated that cystine, rather than the aromatic amino acids in the mycelium of *P. flavo-glaucum*, was the first limiting factor in the growth of rats.

6. It is concluded that all the "essential" amino acids were synthesized by *P. flavo-glaucum* from inorganic nitrogen, but that cystine was present in only small amounts. Both by chemical tests and animal feeding experiments, tyrosine and tryptophane were shown to be synthesized by this species from inorganic nitrogen.

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A NOTE ON THE RICHARDS-JAHN PHOTOELECTRIC NEPHELOMETER[1]

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The photoelectric nephelometer described by Richards and Jahn (1933) for estimating the population density of suspensions of microörganisms is a useful device adaptable to many problems. It is most important, however, to calibrate the instrument first, using the specific microörganisms in question and the conditions under which they are later to be measured. In following growth cycles with the nephelometer, the necessity for taking into consideration changes in size and cytological content of the cells during the various phases has already been mentioned by the authors cited.

The first step in calibration is to determine, in a given suspension, the relationship between numbers of organisms and light absorbed. The present note is concerned only with light absorption relationships in cultures of a given age.

Richards and Jahn state: "The light absorbed [by the cell suspension] is directly proportional to the number of cells. . . . ." Apparently this is only partially true. In calibrating our model of this nephelometer over a wide range of concentrations, using suspensions of bacteria and of barium sulfate, we found a critical point in this relationship, the slope of the line relating total numbers (strictly, total volume) of cells (or particles) with light absorbed changing at a point equivalent to about 100,000,000 *Bacterium coli* per milliliter. The angle between these lines was the same for various bacteria and for barium sulfate, even

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[1] Contribution No. 28 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

though the slopes differed considerably with the several suspensions.

This condition is illustrated in figure 1, obtained with suspensions of *B. coli* and of barium sulfate. The bacterial suspension was a twenty-four-hour broth culture, containing about 200,000,000 cells per milliliter as determined by plate count. (Concentrations of cells much greater than this figure are too dense to be determined with the nephelometer directly, because of its limited range. Furthermore, rapid settling of the particles would preclude accuracy in making readings.) The barium sulfate was made up (Peskett, 1927) to the same turbidity as this culture; both were then serially diluted and readings taken on the nephelometer.

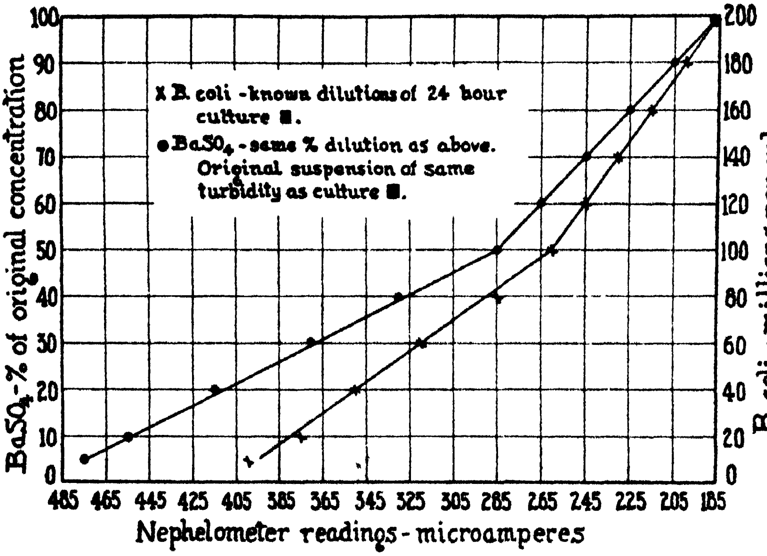

FIG. 1. RELATION BETWEEN LIGHT ABSORBED AND CONCENTRATION OF CELLS (OR PARTICLES) IN VARIOUS DILUTIONS OF STANDARD SUSPENSIONS OF B. COLI AND OF BARIUM SULFATE

In practice it is simple to correct for this change in slope by diluting cultures which contain more than 100,000,000 bacteria per milliliter (equivalent, in this case, to a reading of about 300 microamperes on the nephelometer) to contain fewer cells per unit volume.

The explanation of this critical condition is not entirely obvious. Apparently it is a physical phenomenon inherent in the suspension, possibly involving relations between absorption and reflection of light by the particles (Smith, 1931–1932). In certain cases, at least, the condition may exist, and the apparatus must accordingly be carefully standardized.

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ON THE PLEOMORPHISM OF BACTERIA

I. ON THE PLEOMORPHISM OF B. PARATYPHI B.

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During the last decade, through the publications of Almquist, Löhnis and Enderlein, bacteriological thought has been led again, though along a new path, towards Naegeli's conception of pleomorphism, with the result that the Cohn-Koch doctrine of monomorphism of species is no longer generally accepted.

The statements of the authors mentioned above, and of others who have studied the external features of the bacterial cell, have either called forth objection on the ground that the data might be interpreted in a sense different from pleomorphism (Kuhn), or have excited severe criticism (Neufeld). On the other hand, a series of studies begun by Barthlein and continued by Weil, Arkwright, Andrewes, Hadley and others, investigations which were especially stimulated by d'Herelle's discovery of bacteriophagy, have received general acceptance.

This is somewhat paradoxical, for the works of these latter authors have provided abundant facts favorable to the doctrine of pleomorphism. They have, however, received more attention from the standpoint of variation in physiological characters and in the biochemical (antigenic) nature of the bacterial species, than from the standpoint of morphological pleomorphism, i.e., variability in the external features of the bacterial cell.

In fact Almquist, Löhnis and Enderlein directed their attention to the individual bacterial cell, whereas the authors cited above have been more concerned with aggregates of cells in colonies and cultures. These investigations of variations in bacterial colonies (so-called dissociation or cyclogeny) have enriched bacteriology with a large number of facts essential to the doctrine of pleomorphism in bacteria. However, the above authors have paid little attention to the problem of morphological pleomorphism of the bacterial cell as such, except that some observations have been made on the organs of motility and on capsules.

There is no doubt, however, that a study of the bacterial individual must yield facts of extreme importance. Pokrovskaja's ingenious investigations on this subject have proved with absolute certainty the existence of nuclei in bacteria, and this has been confirmed by Lugovaja and Lebedeva and by Korobkova. Although Pokrovskaja paid attention chiefly to the question of the existence of nuclei in bacteria, her method of investigation opens a new field in the study of the morphology and life of the bacterial cell.

After studying this work, we concluded that an investigation of secondary colonies by Pokrovskaja's method would probably prove the occurrence of pleomorphism in bacteria, and reveal facts sufficient to contradict Cohn and Koch's views on morphological monomorphism of bacterial species. This doctrine has played a most important part in the development of our science, but its authority has too long prevailed over microbiological thought and has prevented free progress.

Accordingly, we undertook an investigation of secondary colonies of *B. paratyphi B* (Schottmuller), which, as is known, are regularly formed on agar containing 1 to 2 per cent raffinose in eighteen to twenty-four hours at 37°, and in twenty-four to seventy-two hours at room temperature. These secondary colonies were examined according to Pokrovskaja's method with but little variation. Impression preparations were made on coverslips, from colonies in Petri dish cultures. They were dried at room temperature, then fixed for thirty minutes in Nikoforoff's mixture, again dried, and stained twenty-four hours in an aqueous solution of Giemsa stain (1 drop to 1 ccm.). This done, they were washed in distilled water, dried, and mounted in cedar oil.

PROTOCOLS

Impression preparation of a secondary colony two days old. The preparation contains rounded cells of various sizes, which are mostly stained pale blue. No definite nucleus may be discerned. A large pale blue body surrounded by a narrow zone of protoplasm stained a somewhat deeper blue is present (fig. 1).

In addition to these pale blue spherical forms, some parts of the colony more rarely contain similar forms stained deep blue, and of these some display a more differentiated nucleus and protoplasm.

On the other hand, enormous numbers of regular bacilli, both regularly and irregularly stained, are present together with the above-mentioned spherical forms. These are of a size typical for the species (fig. 1).

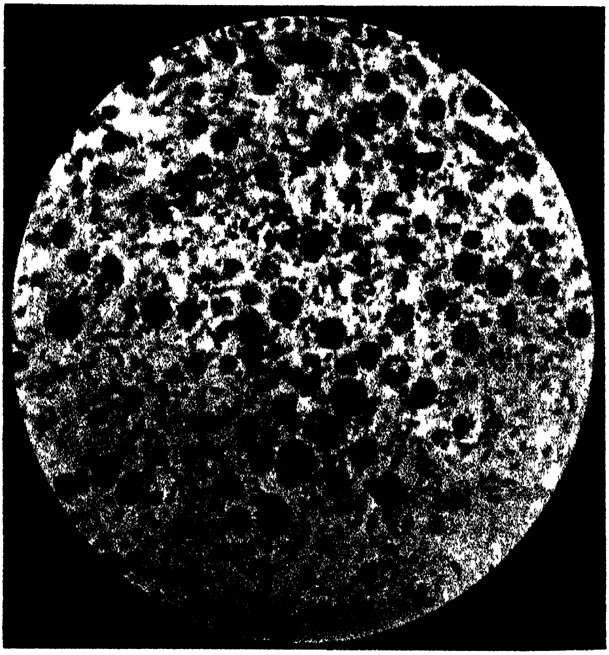

Fig 1

Impression preparation of a secondary colony four days old. This colony contains spherical and oval forms similar to those in the preceding colony, among which a rather large number of bacillary individuals are present. It is as difficult to distinguish the nucleus from the protoplasm as in the first case. Sometimes rope and spindle-like forms are present.

Impression preparation of a secondary colony five days old. Large numbers of spherical and partly oval forms, mostly stained a deep blue, are scattered among common bacillary individuals. A definite nucleus and protoplasm are discernible only in a few cells.

Some parts of the colony display a gradual change of the picture, as some fields contain not only the forms mentioned above, but also rope-, spindle- (fig. 2), and trypanosome- (fig. 3) like individuals, some of which possess a nucleus. In other sections the latter have almost replaced the spherical, oval, and common bacillary forms (fig. 3).

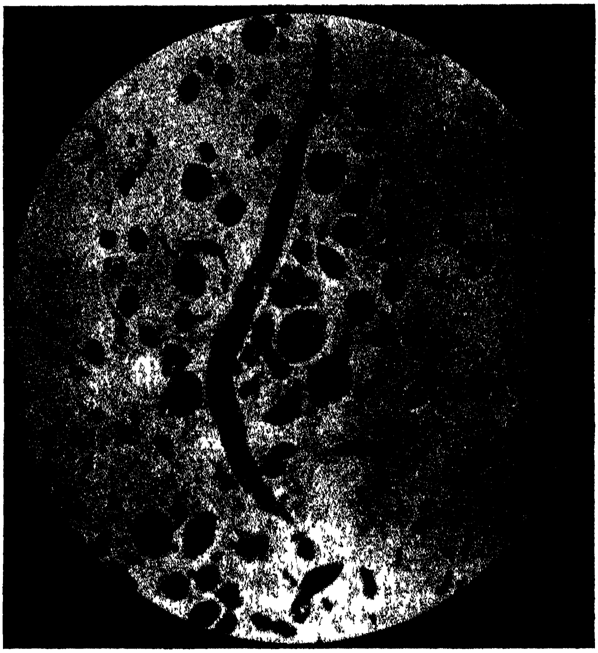

FIG. 2

Impression preparation of a secondary colony six days old. This preparation contains round, partly oval, forms of varying size, some of which are very large. Only a few possess a distinct nucleus, separated from the protoplasm. Almost half of the oval and spherical forms are stained a light blue, whereas the other types have taken a deep violet-blue tint. Among these, considerable numbers of common bacillary forms may be seen.

Impression preparation of a secondary colony eight days old. The bacteria are present mostly as spherical, oval, or irregular polygonal forms. At this stage the majority of cells have a distinct violet, or violet-blue nucleus. This is either round or oval, or irregular in form, quiescent or dividing (figs. 4, 5, 6, 7). The protoplasm may or may not be involved in division. Rope- and spindle-like forms are also present, though not in large numbers. Towards the periphery of the colony the forms described are replaced by nucleated cylindrical cells which in turn give place to the common bacillary forms.

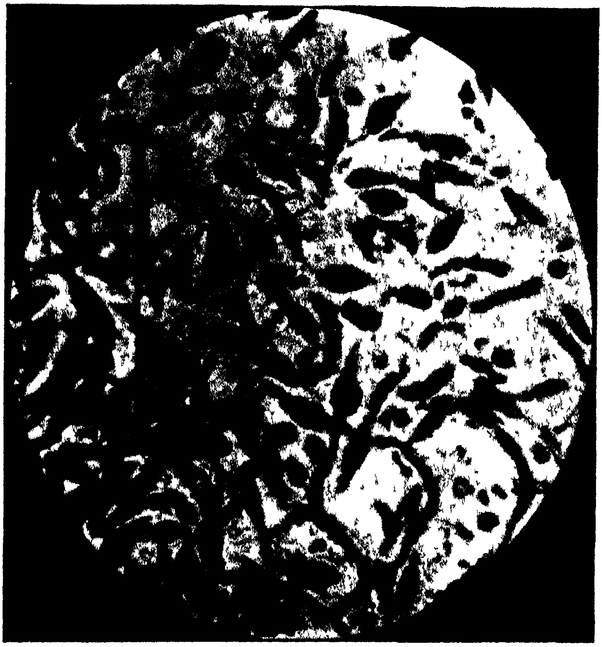

FIG. 3

Impression preparation of a secondary colony nine days old. Spherical or oval cells appear in the centre of the colony. They are sometimes swollen in the middle, and towards the periphery of the colony become elongated. They often exhibit a well-differentiated nucleus and protoplasm. Many common bacillary forms are present.

A smear preparation from the same colony shows common bacillary forms.

Impression preparation of a secondary colony ten days old. Spherical and oval forms are present. These are often swollen in the middle and tapering towards the ends, sometimes trypanosome-like (figs. 8 and 9). They vary in size and often possess easily discernible violet-red nuclei (fig. 9). Some cells are without nuclei, or contain doubtful bodies which cannot be recognized as nuclei. The nucleated cells are quite variable, often bizarre, in form (fig. 9). Together with these large forms, the colony more rarely contains gigantic cells, within which are found bodies of some sort that stain pink with eosin (figs. 10, 11). In addition, long thread-like forms of varying diameter are present, sometimes taking the form of spirochaetes (fig. 12).

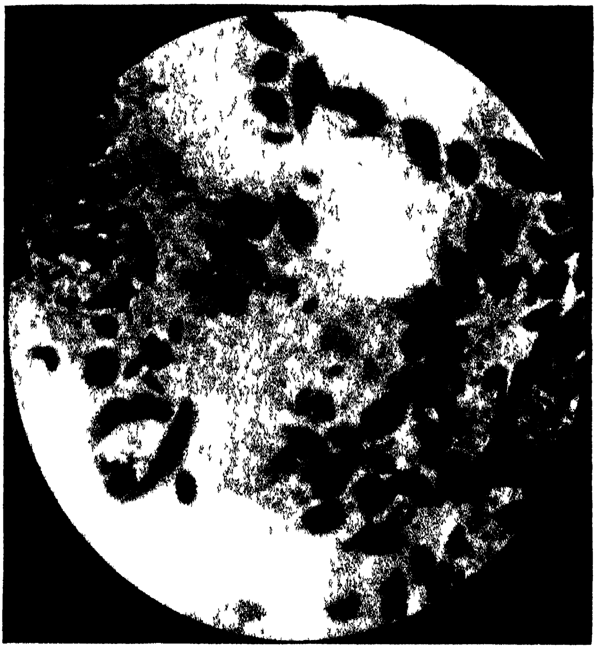

FIG. 4

Fig. 5

Fig. 6

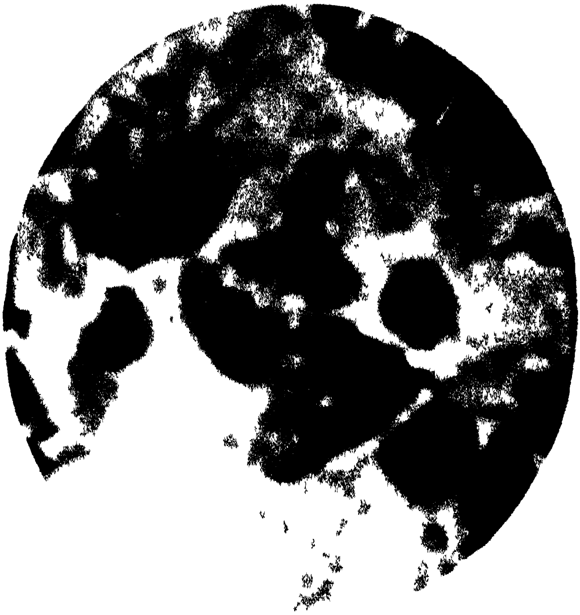

FIG 7

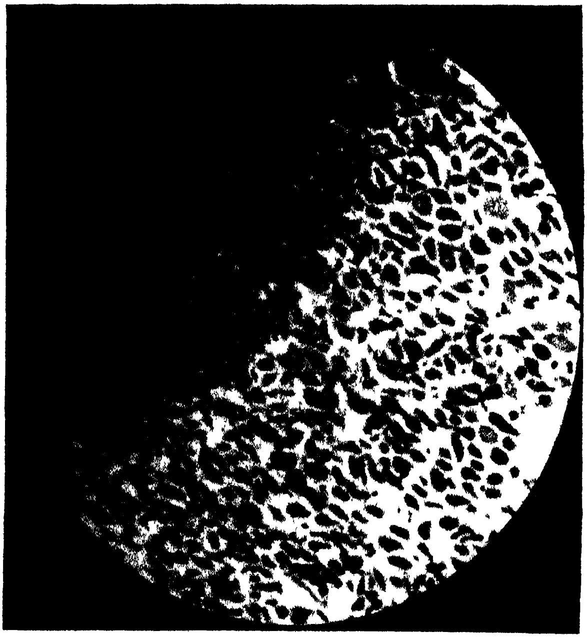

FIG 8

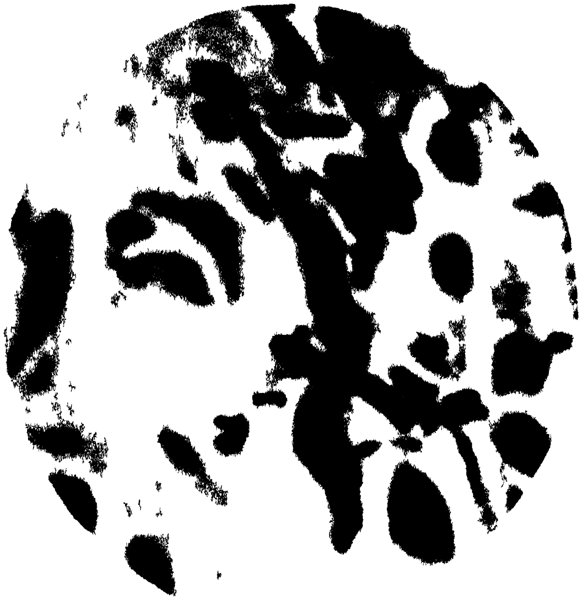

Fig 9

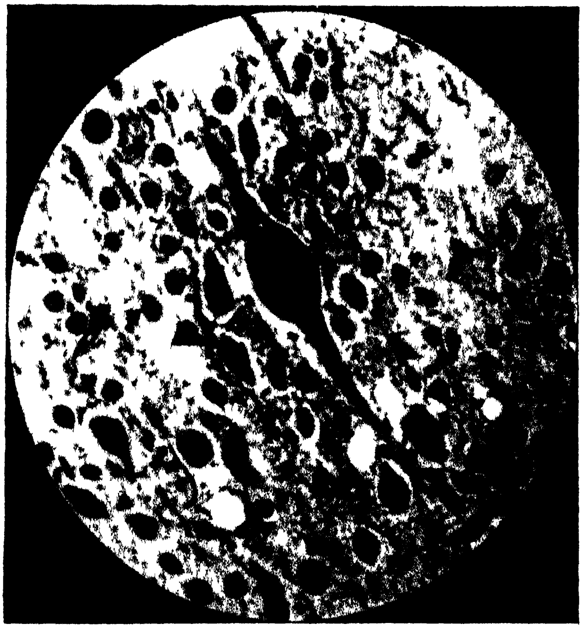

Fig 10

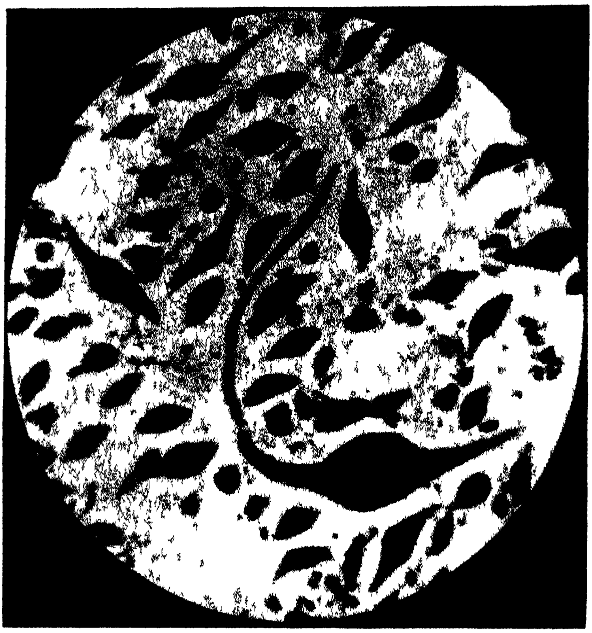

Fig 11

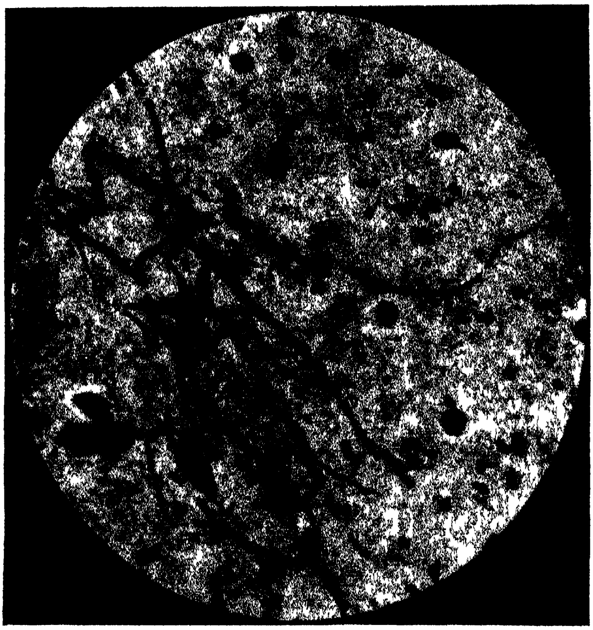

Fig 12

Scattered among the forms described may be found peculiar cylindrical forms resembling the normal *B. paratyphi B*, but apparently containing a differentiated nucleus, as well as some common bacillary forms.

A smear preparation from the same colony shows the ordinary bacillary forms. After studying the impression preparation, however, one may discern amongst these common forms the scarcely visible remnants of the forms which were conspicuous in the impression preparation.

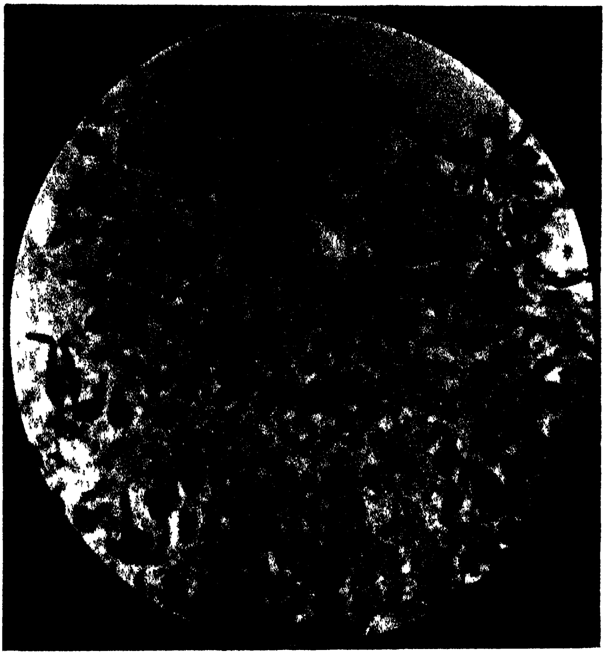

Fig 13

Impression preparation of a secondary colony twelve days old. The common bacillary forms are scarce. Spherical or oval micro-organisms, swollen in the middle and tapering towards the ends predominate. They are spindle-like and mostly very large. The great majority have no nucleus, although there are cells in which a nucleus-like body or corpuscle is present.

Impression preparation of a secondary colony fourteen days old.

The bizarre forms which appeared in earlier colonies are now reduced in number, and a distinctly differentiated nucleus and protoplasm is no longer visible. Small numbers of the bizarre forms are imbedded in an almost structureless mass, which results from a decomposition of these same forms (Löhnis's symplasm). Large numbers of the common bacillary forms occupy the periphery of the colony.

Impression preparation of secondary colonies seventeen and nineteen days old. The greater part of the colony is transformed into an amorphous (or almost amorphous) mass, consisting of dead or dying spherical and oval cells, showing reticulated protoplasm and sometimes nuclei broken up into chromatin granules (caryorhexis) (fig. 13). The outlines of these forms are often lost in the surrounding mass. This amorphous material often contains gigantic trypanosome-like forms (fig. 14). Other fields show only cylindrical forms of varying length, approaching the common bacillary form of the paratyphoid bacilli.

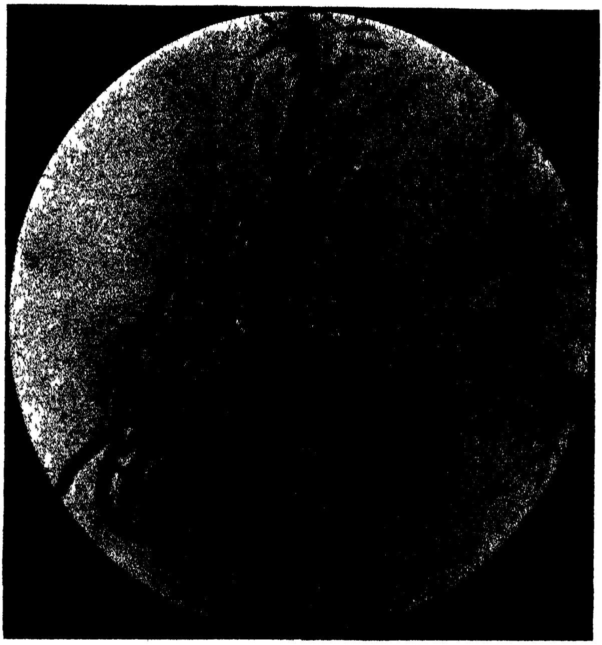

FIG. 14

DISCUSSION

From the protocols and photomicrographs presented, we conclude that the *bacillary form of B. paratyphi B (Schottmuller), although more frequently found, may be considered as only one of many possible forms of existence of this microorganism.*

It may be questionable whether these microorganisms, which vary so greatly in shape, and which arise from secondary colonies, are identical with the original *B. paratyphi B* Schottmüller. But because of the fact that during a given period they reappear in any one of the great number of pure culture colonies investigated, and when transferred to agar slants display the biological and antigenic properties (agglutination up to titre by specific serums) characteristic of *B. paratyphi B*, all possible doubts are removed.

Contrary to what has been believed until now, this organism actually exists in the form of spherical, oval and rectangular cells of varied form. With Giemsa's stain, they are colored either pale blue, blue, or intense blue-violet.

The nucleus either cannot be distinguished from the protoplasm, or it is discernible with great difficulty. In those cases where the nucleus is well differentiated, it is often found *in various stages of division*. *B. paratyphi B*, also assumes barrel-shaped, rope-like and trypanosome-like forms, with or without nuclei. Filamentous and spirochaete-like forms are found, as well as gigantic cells, much larger than those mentioned above. These are barrel-shaped, with long tapering tail-like appendages at their extremities, and filled with some sort of pink-staining granules. Sausage-shaped and cylindrical cells are also found. These are intermediary types, approaching the common form of *B. paratyphi B*, but sometimes they possess a distinct nucleus and are several times larger than the well-known forms of this microbe.

There is no ground for regarding such forms of *B. paratyphi B*, or at least all of them, as abnormal (teratological), injured or dying (degenerating, involutionary) cells.

There seem to be no reasonable grounds for supposing that living, nucleated cells which appear after eight to ten days and sometimes later, are teratological or involutionary, *especially since many of them are undergoing division of both the nucleus and* *the protoplasm.* Such a supposition would be in contradiction of the cytological criteria of decrepit, injured or dying cells. In analyzing the condition of an injured cell, a pathologist takes into consideration the state of the protoplasm as well as that of the nucleus.

However, one might suspect the occurrence of involutionary changes, especially in the later stages of the secondary colonies, when one considers those forms of the microorganism studied which lack a nucleus well differentiated from the protoplasm. But no signs of degeneration could be observed in these latter forms, which, in fact, were already present in the first days of development of the secondary colonies.

We were able, by observing the condition of the protoplasm and of the nucleus, to distinguish degenerated cells from living cells during the later stages of the secondary colonies. The protoplasm of dying paratyphoid bacilli is vacuolated, its affinity for stains is lost, the outlines of cells appear blurred, while the nuclei are in a state of caryokinesis. Dead microörganisms are transformed into an amorphous mass.

It should be explained why previous investigators have not discovered the various forms of *B. paratyphi B*, described in this work. Comparing descriptions and microphotographs of forms found in both impression and smear preparations of colonies aged nine and ten days it appears evident that smears alone yield an incomplete picture. It becomes obvious that the variability of external shape of *B. paratyphi B*, remained unregistered up to now merely because only smears and not impression preparations were in general use. The fact is that during the preparation of smears all other stages of *B. paratyphi B*, except common bacillary forms are destroyed or deformed.

Anybody unfamiliar with the striking pleomorphism of *B. paratyphi B*, would take such remnants for extraneous amorphous dirt. Only the trained eye recognizes in them remnants of destroyed cells.

There are no grounds to think that the pleomorphism described above is a characteristic feature alone of *B. paratyphi B*, Schottmüller.

LÖHNIS, KUHN AND POKROWSKAYA'S PREVIOUS WORKS

The *Pettenkoferia*, which were erroneously interrupted by Kuhn as protozoa parasitic in bacteria proved that pleomorphism existed among other species of bacteria. Nevertheless only the methods of investigation used by Pokrowskaya and ourselves entitle us to state definitely that pleomorphism of bacteria is a proved fact. This is all the more evident since we used nutritive media free of substances which could injure bacteria and cause involution. The forms studied showed enormous variation in external appearance. We are absolutely certain of the vitality of these forms since we were able to observe the condition of the protoplasm and of the nucleus. The purity of our cultures was proved. Any investigator may repeat the experiment and obtain all of the endlessly varied forms, described.

These data enable us to conclude that the doctrine of monomorphism contradicts reality and should be replaced by the statement that bacterial species are pleomorphic. Future investigations must determine whether the various stages of existence of a microbe depend upon certain cycles of development. This problem, as a whole, is of the utmost importance to microbiologists, as is also the question whether pleomorphism occurs in pathogenic microbes within an infected host.

We believe that the facts presented in this paper, in addition to their purely morphological interest, are of greater significance. The morphological pleomorphism of a bacterial individual should influence the morphology of colonies, the multitude of physiological potentialities of the bacterial cell, its antigenic properties,[1] and the co-relations between pathogenic microbes and an individual host organism[2] (infection) on one hand, and populations of host organisms (epizootics and epidemics) on the other.

[1] A series of facts in this category have been discovered as a result of the study of microbic dissociation differences in chemical activity, in susceptibility to bacteriophagy, and in serum reactions between S and R types

[2] Variations in pathogenicity belong in the same category of phenomena It is known that S forms are credited with greater immunizing power and resistance to phagocytosis than R forms.

SUMMARY

1. The bacillary form of *B. paratyphi B*, Schottmüller, though more frequently found, may be regarded as only one of many possible forms of existence of this microörganisms. Because of this, pleomorphism of bacteria should be considered as a proven fact.

2. The doctrine of monomorphism contradicts reality and should be substituted by the statement that bacterial species are pleomorphic.

VARIATIONS IN THE ELECTROPHORETIC MOBILITIES OF THE BRUCELLA GROUPS

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The fact that serological methods fail to furnish any sharp, decisive tests in the differentiation of Brucella strains and that such differentiation now depends on the lesions produced in guinea pigs, the dye and the H_2S test, has made it desirable that another distinguishing character be sought. It was thought that a differentiation might be effected by measuring the zeta-potential or electro-kinetic p.d. resident upon the bacterial surface. Hence the following work was carried out. Dr. Theobald Smith kindly supplied us with strains which he had classified as bovine, porcine and caprine. We have retained his designations.

Quantitative measurements of zeta-potentials have been made on rough and smooth acid-fast bacilli (Kahn and Schwarzkopf, 1931), and rough and smooth intestinal non-flagellate bacteria (Joffe, Hitchcock and Mudd, 1933). A most striking observation was made in the latter group; certain smooth intestinal organisms possess very little if any zeta-potential whereas the rough variants have high zeta-potentials. It was thought, therefore, that some definite relationship between the virulence and cultural differences on the one hand, and the zeta-potential on the other, might exist.

METHODS

The Kunitz (1928) modifications of the Northrop-Kunitz micro-cataphoresis cell was used with a dark field condenser and Bausch and Lomb 8 mm., 0.50 n.a., 21 × objective. Eight readings were taken for each suspension at the two stationary levels, i.e. 0.21 and 0.79 of the inside depth of the cell. Radio B batteries were used. The applied potential of 125 volts gave a gradient through the cell of about 6.5 volts. Electrophoretic mobilities are given in μ per second per volt per centimeter fall in potential along the cell. The electrokinetic p.d. may be calculated from the electrophoretic mobility by multiplying by 12.6, if one assumes the dielectric constant and the viscosity of the suspending medium to be the same as those of pure water at 20°C. Care was taken to measure only those particles which were sharply in focus. Every attempt was made to avoid personal or systematic error. The cell was tested at intervals for symmetry with a suspension of relatively constant electrophoretic mobility.

Bacterial preparations

The organisms were grown on infusion agar slants at pH = 6.8; the cotton plug was burned and a little paraffin was added to insure optimal microaerophilic growth conditions. Loopsful of the growth were rubbed up in 2 or 5 ml. of saline, depending on the amount required for dispersion. The condensation water was carefully avoided (Smith, 1931) to minimize clumping.[1] The organisms were washed twice in NaCl, and once in Walpole's sodium acetate-acetic acid buffer, pH = 5.2, at fiftieth molar concentration. After centrifuging, this first buffer wash-water was removed, and the bacteria were resuspended in the acetate buffer. The electrophoretic mobility was measured in this same solution. *Glassware* was chemically cleaned with concentrated sulfuric acid potassium dichromate cleaning solution, tap water and distilled water.

RESULTS AND DISCUSSION

Results are given in table 1. Measurements of the electrophoretic mobility revealed that except in a few instances, individ-

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[1] Spontaneous agglutination occurs in certain strains of the Brucella group under certain conditions. Human 2a, swine 6a and swine 14, clumped when placed in M/50 acetate buffer. Swine 14 gave the appearance of precipitated or flocculated protein material.

TABLE 1

Electrophoretic mobility in μ per second per volt per centimeter

SOURCE	NUMBER	RACE IDENTIFIED AS	AVERAGE	MAXIMUM	MINIMUM
Human	29	Caprine	3.1	3.6	2.4
	29	Caprine	2.9	3.2	2.4
	29	Caprine	2.9	3.6	2.4
	12	Bovine	2.6	2.9	2.4
	2a*	Porcine	2.0	2.4	1.8
	6	Porcine	2.0	2.9	0.7
	30a	Caprine	1.9	2.4	1.4
	16	Caprine	1.9	2.4	1.7
	26	Caprine	1.6	2.0	1.0
	26	Caprine	1.5	2.4	0.9
	35	Caprine	1.5	1.9	0.5 (1.1)
	10	Caprine	1.0	>1.0	0.5
	10	Caprine	0.9	1.4	0.5
	15	Porcine	0.8	1.6	0.6
	35	Caprine	0.7	1.3	0.6
	15	Porcine	0.6	0.9	0.4
	31	Caprine	0.4	0.7	0.2
Goat	4	Caprine	1.0	1.8	0.8
	4	Caprine	1.0	1.4	0.5
	9	Caprine	0.7	1.1	0.5
Swine	12§	Porcine (Danish)	1.3	1.9	0.4
	6a	Porcine	1.2	1.6	1.1
	14†	Porcine	1.2	1.8	0.7
	6a‡	Porcine	0.9	1.2	0.5
	7	Porcine	0.8	1.1	0.6
	10§	Porcine (Danish)	0.8	1.2	0.5
	10§	Porcine (Danish)	0.6	1.0	0.3
Cow	515b	Bovine	0.9	1.2	0.4
	515a	Bovine	0.7	1.2	0.3
	Spain	Bovine	0.7	1.0	0.4
	1373	Bovine	0.7	1.0	0.4
	1465	Bovine	0.7	1.0	0.6
	1051	Bovine	0.6	0.9	0.3
	1798 (2)	Bovine	0.6	0.7	0.3
	1534	Bovine	0.6	1.0	0.3
	1051	Bovine	0.5	0.7	0.2
Horse	3	Bovine?	1.1	1.8	0.6
	3	Bovine?	0.4	0.6	0.3

* Spontaneous agglutination. Shaken to homogenous suspension.

† Clumped macroscopically.

‡ Clumped macroscopically, giving loose floccular appearance.

§ Give no H_2S reaction, unlike our porcine strains.

The figures given are the electrophoretic mobilities in micra per second per volt per centimeter. See text for further explanation.

ual bacteria did not have the same or nearly the same mobility, but varied over a wide range. Consequently a report of the mean electrophoretic mobility of a number of readings, in this instance, has little biological significance. It represents a statistical mean of a sample which may or may not be a true random sample. For this reason the maximum and the minimum mobility are reported for each culture, and the variability may be judged from these figures. Determinations from several subcultures of the same strain occasionally differed.

In order to obtain more constant results, strains *Br. abortus* 1798, 1534 and 1465 were passed through guinea pigs, recovered from the spleen and plated. These presumably smooth strains varied as much after, as before, animal passage. Other than this, there was no attempt to eliminate dissociated forms if present. Such variability of individuals within a single culture has not been found by one of us in numerous observations within the colon-typhoid group, using the same apparatus and equal care in washing.

The values for the electrophoretic mobility obtained may eventually be correlated with the recent work of Wilson and Miles (1932). They conclude that the antigens present in abortus and melitensis strains are similar, but quantitatively different. There is more of the "a" antigen in the abortus than the "m" antigen, and more of the "m" than "a" in the melitensis strains. Such antigenic differences might possibly account for differences in the electrokinetic properties of the bacterial surfaces of closely related strains.

At present it is impossible to say that there is a constant relation between electrokinetic p.d. and virulence or other characteristics in the Brucella group. Since it has been shown in this laboratory that the electrokinetic p.d. of certain rough strains in the intestinal group (colon-typhoid-dysentery) is very much greater than that of the smooth, one might suspect that the cultures were mixed rough and smooth. According to Wilson and Miles, the melitensis strains are more often rough than the abortus strains.[2] There were no very recently isolated melitensis strains available for examination. This problem awaits further work on antigenic structure and spontaneous dissociation in this group.

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[2] Dr. Smith tells us that roughness as exhibited by spontaneous clumping is relatively rare in the genuine bovine types.

It may be that a study of the electrophoretic mobility of strains dissociated into definite and stable variants, or of strains derived from single cells would yield results of theoretical and practical significance. Since the purpose of the authors was to characterize further and more definitely the strains as found ordinarily in the laboratory this has not been done.

SUMMARY AND CONCLUSIONS

The electrophoretic mobilities of certain strains of the Brucella group have been measured. The great variability within a single culture and the overlap of the groups prevents the establishment of any constant relationship between virulence or cultural characteristics and zeta-potentials. On account of the overlap of these groups it is impossible to refer an unknown strain definitely to one group.

In general, the strains of the bovine group exhibit the lowest electrophoretic mobility, and some of the caprine strains from human sources the highest. The porcine strains show an intermediate mobility. Whether there is any difference in the electrophoretic mobility of dissociated strains, as is the case in the colon-typhoid group, we hope to determine shortly.

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CELL INCLUSIONS AND ENDOSPORE FORMATION IN BACILLUS MYCOIDES

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The first detailed description of a method of endospore formation seems to be that of Robert Koch (1876). Since that time numerous workers have studied this problem with the result that various conflicting methods have been described. The present state of general knowledge concerning the cytology of endospore formation is revealed by comparing the accounts given in recent reviews and textbook literature. While there is lack of uniformity in the descriptions two conflicting methods are quite generally described. These may be illustrated by brief quotations.

Conn (1926) says:

> Within the rod which is to bear the spore a small round body appears which is more highly refractive than the rest of the rod and does not take stains as readily. It grows until it becomes as broad or even broader than the parent organism and perhaps half as long. Then the remains of the original organism die away.

This in brief concise form is the view expressed by Robert Koch (1876) for *B. anthracis* and repeated by DeBary (1887) for *B. megatherium.* It is still to be found in many modern textbooks of bacteriology and Cook (1932) in a recent review states that the method described by DeBary could not be bettered.

A different method is clearly stated by Buchanan (1930) who wrote as follows:

> The endospore formation in a bacterial cell is first indicated by the appearance within the cell of highly refractile granules. These tend to collect at one point in the cell and unite to form a larger globule. This increases in size and density and finally surrounds itself with a firm mem-

brane or spore wall. The spore seems to be made by the contraction of the protoplasm with extrusion of water.

A similar view is given by Lehmann and Neumann (1931) in the following language:

> The sporulation of this species (*B. anthracis*) may serve as a type. It starts with a fine granular cloudiness in the previously homogeneous bacterium. Then in place of these finest tiny particles a small number of slightly coarser granules appear which unite among themselves until small roundish spores lie at regular intervals. These gradually become the oval, strongly refractive, ripe spores. These conditions have usually been explained by saying that the spores are formed by the fusion of granules.

This view was expressed by Zopf (1885) and obtained many advocates following the work of Ernst (1888–89) and Babes (1889). It is almost inseparably linked with the conception of a diffuse nucleus in bacterial cells. Many workers have doubted the nuclear nature of the granules in question as well as their fusion to form spores, but the view has been widely taught and accepted.

It is of interest to note that *B. anthracis* which, according to Koch, forms spores by the gradual development of a single small granule may also serve as the type for spore formation by the fusion of many small granules. There is nothing in any of these statements taken singly to indicate that sporulation lacks uniformity, but when all are considered we are obliged to conclude that the process is not uniform even in a single species.

A third theory which dates from the work of Fisher (1891) appears to be the basis for the concluding sentence quoted from Buchanan. This idea originated in the work of Klebs who found that plasmolysed cells of algae might surround the contracted protoplasm with a membrane and later under favorable conditions initiate new growth.

I have recently had occasion to review the literature of spore formation and to study the development of the spores in *B. mycoides*. This study has convinced me that the methods described above are erroneous and do not represent the more accurate studies which have been made of this subject.

Among the first investigators who questioned the view of Koch and DeBary full credit must be given to Peters (1889), who described the process of sporulation in an unidentified organism designated as *Bacillus E*. His observations were confirmed and extended by Klein (1889). The latter described it as "a new method of spore formation." That this method applies to a wide variety of species has been shown by the subsequent work of A. Meyer (1897–99), Grimme (1902), Preisz (1904), Bredemann (1909) and Georgevitch (1910). The list of organisms includes the best known species such as *B. subtilis*, *B. tumescens*, *B. anthracis*, *B. tetani*, *B. cohaerans*, and *B. amylobacter*.

In a recent paper by Bayne-Jones and Petrilli (1932), the essential features of this method are shown to apply also to *B. megatherium* although these writers make no reference to the earlier work. This most recent paper is of special interest because of the unique record of the process obtained by serial photographs of a single sporulating cell.

According to Preisz (1904) who gave the most complete account of this method, the process of sporulation in *B. anthracis* may be divided into three phases:

1. A membrane develops which sets off a clear, fertile, nucleated segment or spore primordium at one pole equal in diameter but not more than one-fifth to one-third the length of the cell.
2. The contents of this primordium increase in density and differentiate to form a "vorspore" consisting of a more or less elongated, dense, refractive, oval body, "the young definitive spore" surrounded by a less dense layer of cytoplasm.
3. The vorspore migrates to the middle of the rod, the granular content of the parent cell disappears and the ripe spore becomes surrounded by a wall derived from the cytoplasmic layer.

A comparison of the observations on cytological changes recorded by Bayne-Jones and Petrilli for *B. megatherium* with the descriptions given by Preisz for *B. anthracis* and by others for various species reveals no marked differences. The photographic record shows very clearly that a polar spore primordium free of refractive granules is formed without fusion of granules. It is impossible to determine from photographs whether or not the primordium is set off by a membrane but the behavior of the granules appears to support the conclusions of Preisz. The photographic record shows that while granules change position within the area outside they are wholly incapable of penetrating into the primordium. The photographic method of study furnishes conclusive evidence that the definitive spore body is formed by rapid condensation of material within the primordium. Earlier workers, especially Meyer (1897), had emphasized the fact that this body results from condensation of substance rather than continued growth of a small refractive granule.

The cytology of spore formation has received but scant attention by recent workers. This might appear to indicate that the subject is so well known that further study is not necessary. The various conflicting descriptions to be found in textbooks and reviews are opposed to such a conclusion. The tendency among such writers is to accept the view of Koch and DeBary or that of Zopf and Migula. The method described by Fisher is less frequently given while that of Peters, Klein, Meyer, Preisz, and others is rarely mentioned.

CYTOLOGICAL OBSERVATIONS

Very young cells of *B. mycoides* stain uniformly with aqueous solutions of aniline dyes while older cells present an alveolar appearance due to the presence of numerous highly refractive, non-stainable granules which resemble oil globules. Since great importance has been attached to these bodies, especially with regard to their relation to endospore and gonidia formation in this and other species, it was deemed essential to determine their nature as fully as possible. Suitable tests for fat, volutin, and glycogen were made.

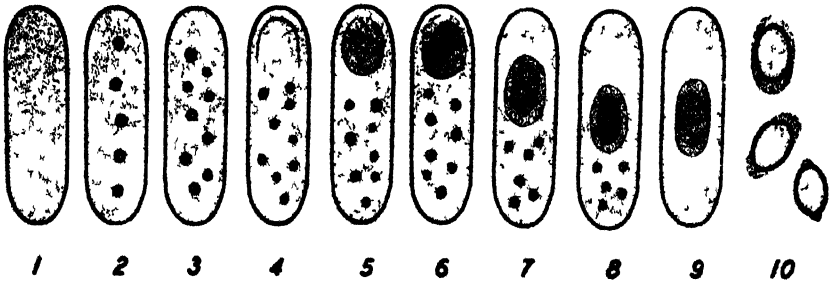
The granules are not stained by aqueous solutions of aniline dyes nor by Gram's method and are not acid fast. They may be stained by Sudan III or dimethylamidoazobenzol and by any of the several methods described by Eisenberg (1909) for staining similar bodies in *B. anthracis*. The most satisfactory results were obtained by the paraphenylenediamin a-naphthol method, by nile blue with the addition of 1 per cent sodium carbonate solution, and by basic fuchsin in combination with various reagents. Wet mounts were found to be more satisfactory than fixed films. The cells were suspended in one of the liquids and allowed to stand one or two minutes. On the addition of the second reagent some precipitation occurred, the amount depending on the strength of the reagents. While this is somewhat troublesome the results are so striking that there need be no confusion.

Preliminary treatment with Lugol's iodine solution, diluted three to five times, followed with diluted aqueous solution of basic fuchsin (0.1 per cent) was found to be a very satisfactory method. The granules take up the dye vigorously and are seen as brilliant red bodies in the faintly stained yellowish-red cytoplasm. By reversing these reagents a slightly different but no less striking differentiation is obtained. The granules which have remained colorless in the fuchsin solution become stained on the addition of iodine, while the cytoplasm gives up the red color and becomes somewhat purple. Picric acid, phenol, platinic chloride, corrosive sublimate or a-naphthol may be used in combination with fuchsin but are generally less satisfactory than iodine. It has not been found possible to stain both the cytoplasm and granules equally by any of these combinations, although the cytoplasm may be counterstained by other dyes.

Tests for volutin and glycogen carried out according to the methods of A. Meyer (1904) and Grimme (1902) gave negative results. On the basis of all reactions it is assumed that the refractive granules consist of non-living substance of a fat-like nature which serves as a food reserve. It should be noted that many workers have regarded similar bodies as consisting of chromatin. Among these may be mentioned Ruzicka (1907), Guilliermond (1908), Swellengrebel (1909) and Schaudinn (1902–3). Their conclusions do not appear to have been based on sufficient or suitable tests.

In order to determine the fate of the granular material and what relation, if any, it bears to spore formation, three typical strains of *B. mycoides* have been studied in detail. The cultures were prepared as giant colonies on the surface of standard agar in petri dishes. At the end of an incubation period of thirty-six to forty-eight hours, cells in all stages of spore formation, as well as young vegetative cells, may be obtained by preparing mounts from the center to the edge of the colony.

The first indication of spore formation to be noted in living unstained cells is the clear polar spore primordium, identical in appearance and doubtless in origin with that of *B. anthracis*, *B. megatherium*, *B. subtilis* and other species which have been described (figs. 4 and 5). The granules are localized in the non-fertile segment of the cell, and there seems to be no evidence that they play any direct part in formation of the primordium. There is no refractile sporogenous granule present in the young primordium and no evidence of shrinkage or plasmolysis of the cell contents.



Figs 1-10

When material of the proper age is examined in dilute fuchsin solution, the primordium is clearly differentiated by its greater staining capacity. Meyer (1899) referred to the primordium as the spore vacuole but the term appears to be objectionable. It is obviously a region of more dense protoplasm such as can be observed throughout the vegetable kingdom in embryonic cells. The cell has been differentiated into two distinct areas, a fertile portion destined to further development as a reproductive structure and a sterile portion containing reserve nutritive material, incapable of further division and destined to die. It has generally been believed that in some manner the nuclear material of the vegetative cell becomes enclosed in the young spore. If this is true it might account for the death of the enucleated segment but this is by no means certain. There is no conclusive evidence that the deeper staining capacity of the spore primordium is due to a difference in chromatic material. On the other hand, such evidence as can be obtained by the stains employed points to the opposite conclusions. The material, except for a single deeply stained granule which is not always visible, has every appearance of uniformly stained cytoplasm. Its greater density indicates a region of greater activity such as may be observed in the tips of hypha threads, young sporangiophores, meristem, or the ooplasm in certain oogamous fungi.

The earliest stages of membrane formation are similar to that described by Preisz (1904) for *B. anthracis*. The membrane extends iris-like from the extreme end of the rod, enclosing all but a thin layer of cytoplasm lying against the original cell wall (fig. 4). The contents continue to increase in density after the membrane has formed (fig. 5). It seems improper, therefore, to speak of the primordium as a structure formed by condensation of material. The greater density is not due to a mere aggregation of material formed elsewhere but to a greater capacity to form new substance.

Just preceding differentiation to form the vorspore the primordium has become quite dense and stains deeply with dilute fuchsin or gentian violet solution (fig. 5). Differentiation within the primordium appears to be a true process of condensation. In unstained living material, a central refractive body forms while the periphery appears as a clear hyaline zone. When stained vitally with dilute gentian violet, the central body while young is more deeply stained than the zone surrounding it (fig. 6). Shades of color extending from deep purple through red to total absence of color may be noted, depending on differences in the stage of development.

Since it is desirable to adopt some suitable terminology for convenience in designating the two parts of the vorspore, the terms endosporium and exosporium are suggested. No evidence has been seen to support the view that the endosporium results from gradual enlargement of a small refractive granule. The photographic record by Bayne-Jones and Petrilli (1932) shows that differentiation within the vorspore is a rapid process. There is every reason to believe that the same is true for all spore-forming species.

The endosporium at the time of differentiation is not strikingly different in size or shape from the ripe spore. It is a somewhat elongated to oval body about two-thirds the diameter of the vorspore. Its position is more often diagonal to the long axis of the rod. The exosporium stains more deeply with aqueous dyes than the remaining cell protoplasm and is Gram-positive (figs. 6, 7, 8, 9).

The vorspore may retain its polar position for some time but ripe spores are invariably located near the middle of the rod (fig. 9). This change in position occurs gradually and appears to be facilitated by withdrawal of cytoplasm toward the sterile pole which leaves a thin clear meniscus in front of the migrating spore (figs. 6, 7). The exosporium becomes somewhat more elongated forming a cylindrical body which is longer than the endosporium but only slightly greater in diameter. It retains its dense staining capacity and at this stage appears in the form of rather large deeply stained hemispherical bodies at the ends of the endosporium while the layer along the sides is quite thin (fig. 9).

Preisz (1904) explained the origin of the ripe spore wall as due to shrinkage of this exosporic layer. This is opposed to our knowledge of the origin of similar spore walls in other plants. The true spore wall is more dense and has little or no capacity for taking stains. Moreover, the exosporic layer may be seen to disappear by a gradual and prolonged process. The cap-like structures at the spore poles become reduced to mere remnants and in the end the ripe spore is naked except for its own true wall which is as refractive and difficult to stain as the spore itself (fig. 10). It is probable that the true spore wall is made up of two layers, the outer exine and the inner intine, as in larger and better known spores but this is difficult to demonstrate. In the not-fully-ripe spore, the exosporium lies outside the exine as a rather thick, easily stainable mass but it is not a part of the true spore wall.

The structure designated here as the vorspore might very appropriately be regarded as the true bacterial sporangium although this term is generally employed to designate the mother cell as a whole. Following this conception, it would be necessary to regard the original cell as a sporongiaphore and the vorspore as an endosporangium. The spore primordium is the young, undifferentiated endosporangium. These suggestions are made for the purpose of emphasizing the true nature of the process rather than to introduce new terminology. It seems probable that in some species the exosporium may persist as a more or less permanent sheath around the spore wall. DeBary (1887) recognized in addition to the true spore membrane a clear refractive lightly contoured layer of a gelatin-like consistency which formed a clear area around the spore and in many cases appeared to be drawn out at one or both poles to form appendages. Similar observations were recorded by others but with no clear interpretation of the origin. Meyer (1897) used the term "plasmareste" to designate foreign bodies which were observed at one or both poles of the fully-ripe free spores. The amount of exosporic material present depends on the age of the spore and it is probable that differences might be noted even in a single species under different methods of cultivation such as liquid or solid media. When the spore has reached maturity, the mother cell contains some weakly-staining cytoplasm which is found principally near the ends of the rod as half-moon shaped areas connected by thin layers along the side walls (fig. 10).

Since the refractive granules do not fuse directly to form a spore the question arises what relation, if any, they bear to sporulation. Both Preisz (1904) and Meyer (1899) observed that granules were either few or absent in ripe sporangia and expressed the opinion that the substance served indirectly as nutritive material for spore building. The photographs presented by Wyckoff and TerLouw (1931) show few or no granules in the spore bearing cells of *B. subtilis*.

So far as it is possible to determine, the granules of *B. mycoides* play no direct part in the early stages of spore formation. They diminish in number during the ripening of the spore and are generally absent in old sporangia. Two explanations may be offered to account for this behavior. The substance may become digested and contribute material for ripening the spore or it may be utilized by the mother cell in its own metabolic processes. It is not impossible that both of these functions are performed, although the latter appears to be less probable. Cells from old broth cultures contain numerous granules but few or no spores. Nyberg (1927) made similar observations and concluded that under conditions of cultivation not suitable for endospore formation, the granules become gonidia. Peters (1889) observed oval spores and spherical refractive bodies in ripe sporangia of Bacillus E but the spherical bodies had no capacity for germination. There is no reason to believe that the refractive granules of *B. mycoides* function as reproductive cells.

A knowledge of the cytological changes involved in spore formation should serve to dispel the confusion which exists as to the true nature of endospores. Kruse (1896) expressed the view that sporulation is comparable to encystment of a protozoal cell for the purpose of resisting unfavorable environmental conditions. This view has had many followers who have insisted that since a vegetative cell produces but a single spore and that upon germination the spore in turn produces one cell, it is better to regard the spore as a vegetative cell in a resting state in which form it is better able to resist unfavorable conditions. Opposed to this conception and supported by abundant cytological evidence is the view that a bacterial spore is a cell set apart for reproduction. Accordingly, it is analogous to the spores of other fungi. Its capacity to resist adverse conditions is shared in common with the great majority of spores throughout the vegetable kingdom. Since the process has every appearance of following a sequence no less definite and orderly than that observed in other groups of fungi, it is logical to conclude that spore forming bacteria share with fungi and other plants the same deep-seated, inherited tendency to form reproductive cells.

SUMMARY

The cells of *B. mycoides*, at the time of spore formation, contain refractive granules which are regarded as cell inclusions of a fat-like nature. Spore formation is not due to fusion of these granules, to the growth of a single small refractive granule, nor to shrinkage of the cell with extrusion of water. The spore arises by differentiation of the contents of a polar primordium which is set off from the remainder of the cell by a membrane. The vorspore consists of an endosporium or definitive body of the spore and a surrounding layer of hyaline cytoplasm, the exosporium. The endosporium develops a true spore wall while the exosporium is gradually reduced to remnants or completely disappears. The refractive granules disappear during the ripening of the spore. They probably contribute material to the later stages of spore development and for metabolic processes of the parent cell.

Endospores are regarded as asexual reproductive bodies analogous to the spores of other fungi.

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SOME PRACTICAL AND THEORETICAL ASPECTS OF MOLD METABOLISM

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Detailed investigation of the metabolism of pure cultures of the lower fungi covers a period of forty years and may be dated from the researches of Wehmer concerning the production of oxalic and citric acids by species of Aspergillus, Penicillium and Citromyces. With gradual improvement in biological technic and with the establishment of order in the difficult classification of the various species of molds there has occurred a widespread interest in mycological chemistry which has been noticeably increased during the past decade. This work has resulted in the establishment of at least three industrial chemical processes in which the biochemical activities of molds are utilized. Various enzyme extracts are now obtained from molds on a large scale and are finding important application in different fields. Investigations concerning the composition of mold mycelia are being pursued in various laboratories and promise to yield results of considerable importance. This paper is limited, however, to a discussion of the production by molds of definite chemical products in the substrates upon which they are cultured.

CITRIC ACID

The production of citric acid from sugars by molds was first investigated by Wehmer (1892) forty years ago. Since that time, particularly during the past fifteen years, the mycological formation of citric acid has been studied intensively (Bernhauer, 1932) and its industrial utilization has been successfully accomplished. Production of citric acid in the United States by the mycological process is considerable, since large amounts are exported in addition to those required by domestic demand. Successful plant operation of the process has also been reported to be under way in Belgium, Czechoslovakia and Russia.

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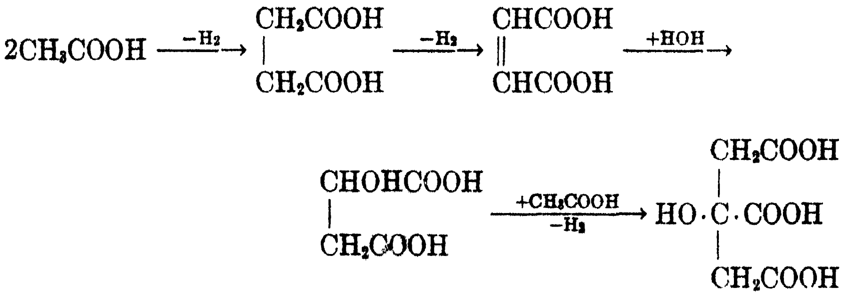
[1] Presented before 35th annual meeting of the Society of American Bacteriologists, Philadelphia, Dec. 27, 1933. 239th contribution from the Division.

Recent investigations have brought forward new viewpoints of the chemical reactions leading to the formation of citric acid from sugars by molds and point to the possibility that the acid may result from a series of reactions analogous to those established in certain yeast and bacteria fermentations. Chrzaszcz and Tiukow (1930, 1932) have demonstrated the ability of certain Penicillia to produce citric acid along with succinic, fumaric and malic acids from ethanol and acetic acid and suggest the following series of reactions to explain the formation of these compounds:

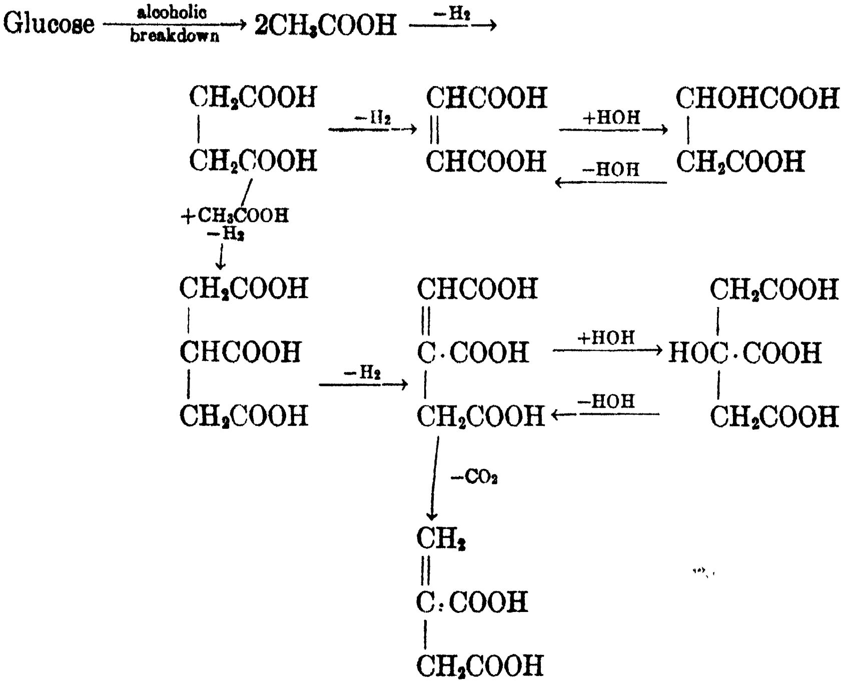


$$2CH_3COOH \xrightarrow{-H_2} \begin{array}{l} CH_2COOH \\ | \\ CH_2COOH \end{array} \xrightarrow{-H_2} \begin{array}{l} CHCOOH \\ \| \\ CHCOOH \end{array} \xrightarrow{+HOH}$$

$$\begin{array}{l} CHOHCOOH \\ | \\ CH_2COOH \end{array} \xrightarrow[-H_2]{+CH_3COOH} \begin{array}{c} CH_2COOH \\ | \\ HO\cdot C\cdot COOH \\ | \\ CH_2COOH \end{array}$$

In this connection it is of interest that the formation of succinic, malic and citric acids from acetic acid has been observed in oxygenated cultures of yeast (Wieland and Sonderhoff, 1932).

Bernhauer, Böckl and Siebenauger (1932) have extended and modified these concepts and have suggested that the formation of ethanol and acetic acid by molds is preceded by an alcoholic breakdown of the sugar according to Neuberg's scheme. This view has been supported by the isolation of methyl glyoxal from sodium hexose diphosphate cultures of citric-acid-producing Aspergilli (Suthers and Walker, 1932). Bernhauer and Siebenauger (1931) have suggested that citric acid is formed from acetic acid by way of aconitic acid in a manner analogous to the succinic-fumaric-malic acid relationships established in enzyme studies of certain bacteria, and they have cited experimental evidence that citric-acid-producing organisms can not only transform aconitic acid to citric acid but can also bring about the reverse reaction. While aconitic acid has not been isolated from sugar cultures of molds it is of interest that itaconic acid has been so recovered in appreciable yields (Kinoshita, 1931). The following series of reactions expresses the mechanism by which citric acid may be formed according to Bernhauer's concept:



$$\text{Glucose} \xrightarrow[\text{breakdown}]{\text{alcoholic}} 2CH_3COOH \xrightarrow{-H_2}$$

$$\begin{array}{c} CH_2COOH \\ | \\ CH_2COOH \end{array} \xrightarrow{-H_2} \begin{array}{c} CHCOOH \\ \| \\ CHCOOH \end{array} \underset{-HOH}{\overset{+HOH}{\rightleftarrows}} \begin{array}{c} CHOHCOOH \\ | \\ CH_2COOH \end{array}$$

$$\downarrow {\scriptstyle +CH_3COOH \atop -H_2}$$

$$\begin{array}{c} CH_2COOH \\ | \\ CHCOOH \\ | \\ CH_2COOH \end{array} \xrightarrow{-H_2} \begin{array}{c} CHCOOH \\ \| \\ C\cdot COOH \\ | \\ CH_2COOH \end{array} \underset{-HOH}{\overset{+HOH}{\rightleftarrows}} \begin{array}{c} CH_2COOH \\ | \\ HOC\cdot COOH \\ | \\ CH_2COOH \end{array}$$

$$\downarrow {\scriptstyle -CO_2}$$

$$\begin{array}{c} CH_2 \\ \| \\ C\cdot COOH \\ | \\ CH_2COOH \end{array}$$

The formation of fumaric and succinic acids by molds is of considerable interest and significance. Butkewitsch and Federoff (1929, 1930) have suggested that succinic acid is formed from acetic acid which in turn is produced by oxidation of ethanol or dismutation of acetaldehyde arising from the breakdown of sugars, basing their statements on experiments carried out with species of Mucor. Fusaria as a rule produce ethanol in appreciable quantities when grown under the usual conditions of surface culture on glucose solutions (Anderson, 1924; Birkinshaw et al., 1931; Letcher and Willaman, 1928) and there is some evidence that its formation follows the usual course found in yeast fermentations (Anderson, Everett and Adams, 1933; White and Willaman, 1928). Unpublished work carried out in the Color and Farm Waste Division with certain species of Fusarium has demonstrated that, if these organisms are grown submerged in well-aerated glucose solutions containing $CaCO_3$, appreciable quantities of succinic acid are formed together with noticeable amounts of acetaldehyde and acetic acid. The quantity of ethanol produced under these conditions is much less than in surface cultures. In this case succinic acid is produced definitely at the expense of the glucose, probably according to the mecha nism proposed by Butkewitsch and Federoff.

The close relationships obtaining in the formation of citric, fumaric, malic and succinic acids and their direct connection with ethanol and acetic acid indicate a common mechanism governing their formation up to a certain point. The preponderance of one or more end-products probably depends on the nature of the enzymic complexes of the particular organism and on the cultural conditions which determine which complex will function most effectively.

GLUCONIC ACID

Although not discovered among the products of mold metabolism until 1922, gluconic acid has since been recognized as a commonly-occurring substance in glucose cultures of molds (Bernhauer, 1932). Rather detailed studies of the oxidation of glucose to gluconic acid, including semi-plant scale investigations, have been carried out in the Color and Farm Waste Division (May et al., 1929). The process is operated in much the same manner as that used for the production of citric acid. Shallow aluminum pans are used for culture vessels and yields of around 60 per cent of theory are readily obtained from 20 to 25 per cent commercial glucose solutions. To overcome the obvious disadvantages inherent in the use of shallow pans some studies have been made of the production of gluconic acid by submerged mold growths in aerated glucose solutions. When the oxidation is carried out under increased air pressures and in the presence of $CaCO_3$, yields of gluconic acid from 80 to 90 per cent of theory are obtained from 20 per cent commercial glucose solutions.

The mechanism governing the formation of gluconic acid is apparently one of simple oxidation. Although the possibility of a Cannizzaro reaction, involving the production of a molecule each of gluconic acid and mannitol from two molecules of glucose, has been pointed out (Raistrick and Rintoul, 1931), the enzyme studies of Müller (1929) indicate that the acid is formed by direct oxidation or dehydrogenation of glucose.

KOJIC ACID

The production of kojic acid from sugars by species of the Aspergillus flavus-oryzae groups is an oxidation process that could be developed readily on an industrial scale in a manner similar to that employed for gluconic and citric acids should sufficient demand warrant it. Yields around 50 per cent of theory may be obtained from 20 to 25 per cent solutions of commercial glucose (May et al., 1931). Kojic acid may be formed by molds from a variety of carbon sources, ranging from starch and inulin to glycerol and dihydroxy acetone. Yields of 30 per cent have been obtained from the last-mentioned compound (Challenger, Klein and Walker, 1931)). As yet no completely acceptable mechanism has been suggested to explain its formation, and it is possible that an initial series of reactions are involved which are similar to those encountered in the production of citric and succinic acids. The flavus-oryzae group of Aspergillus is capable of producing ethanol under certain conditions of culture, indicating that these organisms possess the enzymic system required for the alcoholic breakdown of hexoses. It is of interest that the formation of kojic acid from mannitol by certain acetic acid bacteria has been reported (Takahashi and Asai, 1933).

GALLIC ACID

This acid has long been known to be produced by the action of molds on tannin. In the modern process clear tannin extract is sterilized and inoculated with a pure culture of a species of Aspergillus. The solution is aerated and agitated so that the organism develops in a submerged condition and hydrolysis of the tannin proceeds rapidly (Calmette, 1900). Fernbach (1900) and Potterin (1900) independently of each other demonstrated that the enzyme tannase could be separated from *Aspergillus niger* and could then be used to hydrolyze solutions of tannin to gallic acid.

INDUSTRIAL ALCOHOL

The amylo process for the saccharification of starch, in which a species of Mucor is usually employed, is used to some extent in distilleries instead of the customary malting process. An account of the operation of the amylo process has been published recently (Owen, 1933). The cooked starch is inoculated with the organism and the mash is aerated and agitated. Hydrolysis proceeds rapidly and usually within twenty-four hours has reached a point which permits inoculation with yeast. Alcohol recovery follows the usual distillery practice.

The work of Raistrick and his associates (1931) deserves special mention in any discussion of mold metabolism. These investigators have surveyed systematically the biochemistry of more than two hundred species of molds and have isolated and identified many new compounds resulting from the metabolism of glucose (Birkinshaw and Raistrick, 1933; Charles et al., 1933; Oxford and Raistrick, 1933; Raistrick and Simonart, 1933). As space is too limited for any detailed account of this work, the interested reader is referred to the original publications for particular phases of these investigations.

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THE DISAGREEMENT IN MITOGENETIC EXPERIMENTS, A PROBLEM IN BACTERIAL PHYSIOLOGY[1]

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Fundamentally new scientific discoveries are by no means readily accepted by fellow scientists. They are, as a group, quite conservative, and intolerant toward radically new ideas. In 1867 Lister suggested antiseptic treatment of wounds. The Lancet stated in 1875 that there was less antiseptic surgery practised than ever. In 1880, the gynecologist Tait called Lister's idea the strangest surgical craze of the 19th century. Buchner's discovery of zymase was questioned for a number of years because other German and English biochemists could not obtain the same results, and it took more than five years before it was realized that the variety of yeast, and especially its physiological condition were very important factors. Mitogenetic radiation, proved by hundreds of onion root experiments and by tens of thousands of yeast experiments (Siebert reported in 1930 that he alone had carried out more than 2500 experiments; Kalendaroff made 1459 experiments for the establishment of the nerve spectra, etc.), is still doubted by many biologists.

Two things seem to have made the biologist suspicious. He does not like the conception of radiating organisms. It smacks of the popular conception of radiating personality, miraculous healing powers and similar mystic superstitions. Further, some biologists did not obtain the same results when they repeated the work of the Russian, German, French or Dutch workers.

Let us start with the first objection, the suspicion of mysticism. If biologists would realize the facts, this radiation would not appear mysterious at all. Many of the well-known chemical reactions emit energy. We measure it as calories, record it as heat of reaction: it may be produced as heat, or it may be produced, at least partly, in other forms, e.g. in the form of visible light, as in the slow oxidation of phosphorus, or in the light produced by luminescent bacteria.

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Recent investigations show that a number of chemical reactions emit ultraviolet light, too weak to blacken the photographic plate, but strong enough to be detected by the most accurate physical instrument, the Geiger Counter which is an extremely sensitive photo-electric cell. Frank and Rodionow in Russia (1932), Gerlach in Germany (1933), Audubert in France (1933) demonstrated ultraviolet radiations from inorganic and organic oxidations.

The wave length has not been measured accurately, but it has been shown that the wave lengths between 2000 and 2700 Å are contained in this radiation. The emanation of short-wave ultraviolet light from chemical reactions cannot be doubted.

A large number of experiments have been performed successfully to show that monochromatic light in this spectral region obtained from physical sources (arc, spark, glow discharge) has a very decided influence upon the growth rate of yeasts, of bacteria, of onion roots, and other organisms or tissues. The most comprehensive investigation is that by Chariton, Frank and Kannegiesser (1930) on the growth rate of yeast. By varying the intensity as well as the wave length, a definite stimulating region could be established which begins near 2600 Å, and whose lower range is limited by the absorption of ultraviolet radiation in air, quartz and water.

Radiations from chemical reactions show the same effects. Increased growth rates have been obtained by the radiations from such simple reactions as acid + alkali (Wolff and Ras, 1932), or pyrogallic acid + O_2, or from alcoholic fermentation or protein digestion (Karpass and Lanschina, 1929).

Since chemical and biochemical reactions emit ultraviolet rays of a wave length between 1900 and 2700 Å, and since emission in this range stimulates the growth rate of certain organisms, it would be strange indeed if the same processes did not cause the same effects when occurring in the living organism. If living cells with an active metabolism do not radiate, it would be necessary to explain why they do not; according to physico-chemical experience, they should.

There is nothing at all mystical about ultraviolet rays from organisms, and we can proceed to the second point of the discussion, the inability of some biologists to reproduce the results which have led to the assumption of biological radiation. We are dealing with a new property of living organisms which is by no means well understood. Let us omit the old onion root method which has only historical interest at the present time (Summary of the earlier work by Gurwitsch, 1929), and let us consider the growth rate of bacteria, and the rate of bud formation of yeast. These organisms are easy to grow, but when growth rates are to be compared, a fairly definite understanding of the measurement of growth rates, of the lag phase, of differences between old and young cells is necessary for reliable experiments and correct interpretation.

The Russian scientists who first used yeasts and bacteria as detectors for mitogenetic rays were no bacteriologists; this is very evident from their directions for making media, and their description of procedure. Nothing is said about the physiological state of the cell, though they soon observed that the cells did not react during their entire growth period. Baron (1930) discovered anew the lag phase of yeast growth with which he was not familiar. Hence his instruction that the yeast should be grown for six to ten hours at room temperature in order to respond readily. Unfortunately, it was not stated that 12°C. is considered a normal room temperature in Moscow and Leningrad, and investigators following such directions literally, and at American room temperatures, would doubtless have obtained an entirely different physiological stage.

The directions sound quite different when given by a bacteriologist. Wolff and Ras, in their first publication (1931) stated very plainly and definitely that bacteria respond to radiation only during the lag phase.

Several other factors are very important, and have been responsible for negative results. The radiation is extremely weak, far below the sensitivity of photographic plates. Light of this wave length is very readily absorbed by the ordinary culture media. A layer of standard broth 1 mm. thick absorbs all mitogenetic rays. Ferguson and Rahn (1933) used broth diluted 1:10 for all experiments with bacteria.

It is also frequently overlooked that over-exposure obliterates the growth acceleration; the exposure time is therefore very important, and Wolff and Ras (1933) stated recently that most of the negative results are probably due to over-exposure.

Aside from these definitely established facts, there are many unknown stumbling blocks. For example, it was recently shown by Ruyssen (1933) that the flame of the Bunsen burner is a strong source of mitogenetic rays; at 3 cm. from the flame, an exposure of fifteen to thirty seconds gives strong mitogenetic effects. If the radiation from a Bunsen burner falls upon the bacteria during transfer, it may stimulate the controls, as well as the culture to be exposed, and there would be no difference in growth rates—which would be interpreted as showing no effect.

The growth rate of bacteria is usually determined by plate count or by nephelometric turbidity measurements, and there is little chance for mistakes in these methods. However, when the growth rate of yeast is measured by the percentage of cells showing buds, errors can creep in. When a yeast culture is growing at a normal rate, after the lag phase has been overcome, the percentage of buds is constant (Tuthill and Rahn, 1933). It depends upon the species of yeast, but is independent of the growth rate, of temperature, of food, etc. If at this stage, the growth rate were accelerated by irradiation, it could not possibly affect the percentage of buds. Only when old yeast cells, without buds, are transferred to a fresh medium, will there be a gradual increase in buds, and during this stage, an increase in the growth rate would show itself by a larger percentage of buds. As a matter of fact, yeast at this stage reacts very promptly towards mitogenetic rays. Here again, good response is obtained only during the lag phase of growth. The lag period of a one day old culture is shorter than that of a six-day-old culture, and the moment of irradiation must be chosen accordingly.

It will be easily seen from these accounts that negative results cannot be very surprising so long as we know so little about this radiation. But several investigators have claimed that their negative results disprove the positive results of others. That is a fallacy. When two investigators obtain different results, it does not prove that one has been right and the other wrong, it proves only that they did not make the same experiment, that somewhere the conditions were different. In order to disprove mitogenetic radiation, it would be necessary to repeat the same experiments with the same results and then show that the mitogenetic effect is not due to radiation, but to some other factor. The mitogenetic effect has been proved to be a fact by many thousand experiments. No new experiments can undo these facts; they can only change their interpretation.

The absence of the mitogenetic effect may be due to any of the known causes, or to some unknown influences. Even in the laboratories which have been working on biological radiations for several years, it occurs occasionally that no effects can be obtained even for weeks in succession. The reason is not known, and aside from patience, the only thing that can be done is a change of the culture of the detector organism.

How the short ultraviolet rays affect the cell to accelerate growth is now known. The intensity is so weak that the energy gain must be negligible. It must be that some process is released which, after release, continues spontaneously. It is suggestive that bacteria and yeasts respond most readily, perhaps exclusively, during the lag phase. There may be some phase in the rejuvenation of the old resting cells into young, actively growing cells which is speeded up by this weak ultra-violet light. Mitogenetic rays are bound to play an important rôle in growth processes of all organisms, but the solution of the fundamental problems, the explanation of the effect will probably be accomplished in the realm of bacteriology.

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THE GROWTH AND METABOLISM OF MIXED CULTURES OF AMMONIA-PRODUCING MICROÖRGANISMS[1]

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The microbial decomposition of organic materials in the soil is a dynamic process, the speed of which is governed (among other things) by the substrate upon which the microörganisms act. The substrate in nitrification is ammonia which is produced in the process of ammonification. Past work from this laboratory (Greaves, 1927) has shown that nitrate production may be defined by Robertson's monomolecular autocatalytic equation (Pulley and Greaves, 1932). This report presents further studies of the growth and metabolism of mixed cultures of ammonifying microorganisms from various soils.

Four soils were used: (1) College Farm soil, a highly-productive calcareous sandy loam, low in organic carbon and nitrogen but otherwise well-supplied with the essential elements of plant-food; (2) Benson soil, a sandy loam containing more clay than the College Farm soil and sufficient alkali carbonates to render it barren; (3) Corinne alkali soil, a fine sandy loam containing sufficient chlorides to render it barren except for a few salt-tolerant plants; and (4) Richland Acres soil, a fine sandy loam made barren by its great sulfate content. In addition to these, the College Farm soil was made "alkali" by adding various salts. All alkali soils had been leached before using, but they still contained sufficient salts to reduce their productivity greatly. The ammonia was determined by seeding 200-gram portions of 1 per cent sterile peptone solution with 1 gram of the soil. All soil cultures were incubated at 28° to 30°C. Periodically, at twelve-hour intervals, 10-cc. portions were withdrawn under aseptic conditions and the ammonia determined. Using a duplicate set of solutions, total numbers were determined at twelve-hour intervals by plating 1 cc. of the peptone solution on peptone agar-agar and counting the number of colonies which had developed at the end of seven days. Results reported for numbers, as well as milligrams of ammonia produced, are the averages of four or more determinations made on separately incubated peptone solutions.

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[1] Contribution from Department of Chemistry and Bacteriology, Utah Agricultural Experiment Station. Publication authorized by Director, April 7, 1934.

[2] Chemist and Bacteriologist, and graduate student, respectively.

An attempt was first made to apply Robertson's autocatalytic growth formula (1923) to the ammonification data. It was soon apparent that the data deviated markedly from this curve during the first twenty-four to thirty-six hours. In fact, the lower asymptote of the curve was not zero but a negative quantity below the origin. Pearl and Reed (1920; 1922; 1924) likewise have found it necessary to modify their original logistic curve, placing the lower asymptote *d* units below the origin. In the work, reported in this article, the authors have used the modified growth curve, the formula of which is:

$$(1) \qquad y - d = \frac{K}{1 + Ce^{rt}}$$

where y denotes milligrams of ammonia accumulated in the time (t); K,C, and r are constants. The constants for the different curves were evaluated in the following manner: Equation (1) can readily be converted into the form:

$$(2) \qquad \log_e \frac{K - (y - d)}{(y - d)} = \log_e C + rt$$

From this it follows that $\frac{K - (y - d)}{(y - d)}$ must be a straight line function of the time (t). As the method of least squares cannot be applied to the fitting of a curve in which, as in the logistic curve, the constants to be determined enter the expression in other than a linear manner, the easiest way to fit the data is to make as satisfactory an estimate as possible as to the value of the upper and lower asymptotes of the curve. This procedure has been followed in evaluating all the constants.

The lower asymptote gives the value of d direct, while the value of K is obtained by subtracting the value of d from the upper asymptote. The value

$$Z = \frac{K - (y - d)}{(y - d)}$$

was calculated for each observation. The values of Z thus obtained were plotted against the corresponding time on the abscissa on arithlog paper. If estimates for the values of d and K were satisfactory, the values of Z fell nearly in a straight line. If not, new values of d and K were tried until a straight line was obtained. The slope m of the line thus determined was calculated from any two convenient points on the line. The value of Z when $t = 0$ is equal to Z_0, which, on the arithlog paper, is read directly as C; r is simply 2.303 m.

The ammonification data on the eleven samples of soil used in this work have been treated in this manner. These data, from six of the soils, together with the theoretical curves and the formula, are given in figures 1 to 3, inclusive. It is obvious that the rate of ammonification in these soils is closely approximated by this type of curve.

The equation constants obtained from the different soils are given in table 1. The value $K - d$ is a measure of the maximum ammonia accumulation and does not vary significantly between the different soils, with the exception of the Benson soil which has the low value of 108, probably due to the heaviness of the soil together with the high sodium-carbonate content.

Leaching increases the slope of the curve as reflected in the increase in the value of r. Adding sodium chloride has little effect. The addition of sulfates or carbonates markedly decreased the slope of the curve, as shown by the value of r. When both sodium carbonate and sodium sulfate were added to the soil, the slope of the curve was even greater than the slope of the

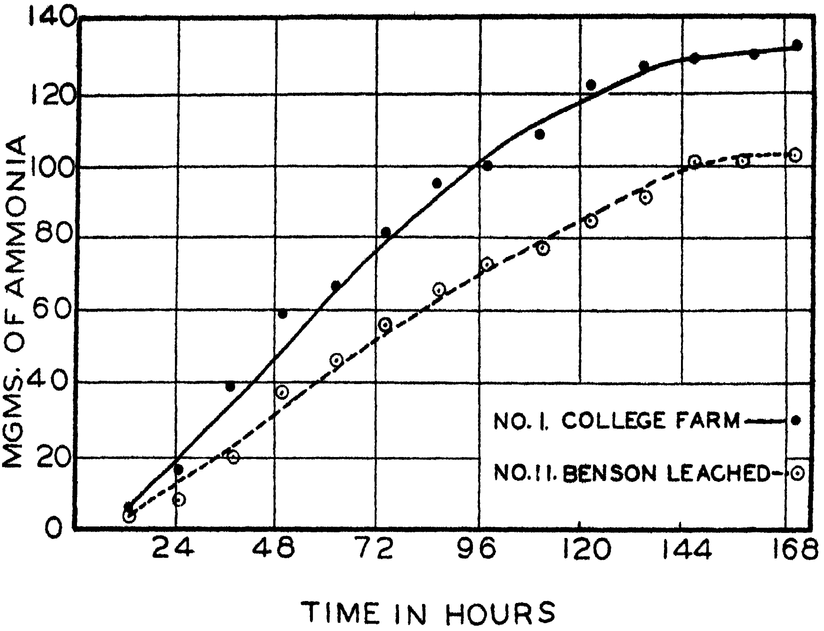
140
120
100
80
60
40
20
0
MGMS. OF AMMONIA
24
48
72
96
120
144
168
TIME IN HOURS
NO. I. COLLEGE FARM ●
NO. 11. BENSON LEACHED ⊙

FIG. 1

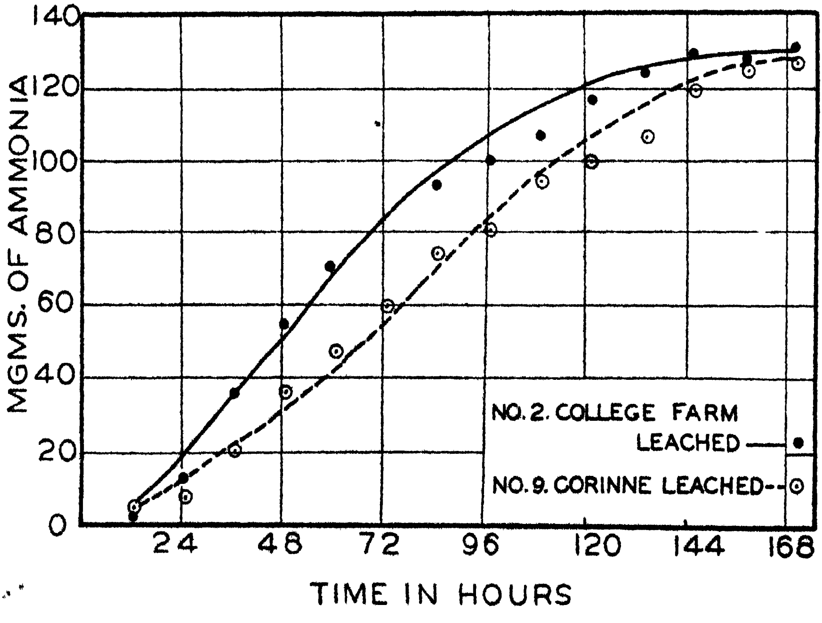
140
120
100
80
60
40
20
0
MGMS. OF AMMONIA
24
48
72
96
120
144
168
TIME IN HOURS
NO. 2. COLLEGE FARM LEACHED ●
NO. 9. CORINNE LEACHED ⊙

FIG. 2

leached College Farm soil. The addition of all three salts decreased the value of *r*. The marked effect of sodium carbonate

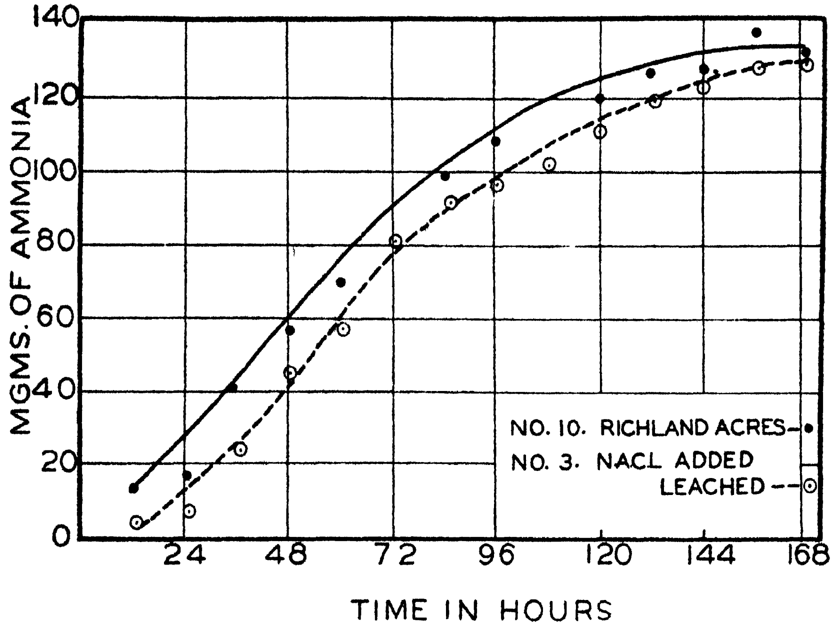

FIG. 3

TABLE 1

Equation constants obtained from the ammonification of the eleven soils

SOIL USED	*K*	*d*	*r*	*C*	*K + d*
College Farm	170	−35	−0.031	4.70	135
College Farm, leached	156	−26	−0.037	6.00	130
NaCl, added leached	158	−25	−0.035	4.80	133
Na_2SO_4, added leached	182	−49	−0.027	2.95	133
Na_2CO_3, added leached	169	−32	−0.027	4.95	137
NaCl—Na_2SO_4, leached	165	−30.5	−0.026	3.95	134.5
Na_2SO_4—Na_2CO_3, leached	143	−15	−0.038	8.15	128
NaCl—Na_2SO_4—Na_2CO_3, added leached	165	−34	−0.028	4.85	131
Corinne, leached	153	−15	−0.030	9.80	138
Richland Acres, leached	163	−31	−0.032	6.00	132
Benson, leached	148	−40	−0.023	3.22	108

is shown by the low value of *r* obtained in the sodium-carbonate rich Benson-leached soil.

The logarithms of the number of bacteria found in 1 cc. of peptone solutions after varying incubation periods are given in table 2.

The number of bacteria carried into the peptone solution with the 1 gram of soil varied from 6000 to 60,000. It was lowest in the case of the Richland Acres and highest in the case of the College Farm soil. All showed a lag period during the first twenty-four hours. This was least perceptible in the sodium-sulfate soil and greatest in the non-alkali soil, which may indicate that the lag phenomenon is associated with osmotic disturbances. The number of organisms rapidly increased up to 108 hours when the solutions were carrying from 70 to 350 million organisms which would grow on the peptone agar-agar. From this period on to the end of the experiment there was usually a gradual decrease in the number of microörganisms present. The outstanding factor appearing in all the solutions was the rapidity of multiplication of the organisms coming from the alkali soil. Although alkali soils contain few microörganisms, when these microörganisms are placed in appropriate media they rapidly multiply.

The great variation from period to period in the number of organisms makes it impractical to attempt to obtain a mathematical statement for growth. However, it is interesting to obtain a correlation between total numbers counted in the progress of the reaction and ammonia produced. This has been calculated according to the formula

$$r_{12} = \Sigma \frac{(x_1 x_2)}{N \sigma_1 \sigma_2}$$

where r_{12} is the correlation between the two variables X_1 and X_2, of which σ_1 and σ_2 are the respective standard deviations and n is the number of pairs of variables; x_1 and x_2 are deviations from the means of X_1 and X_2, respectively. The probable error has been calculated according to the formula:

$$\text{P.E.}_r = .67449 \frac{1 - r^2}{\sqrt{N}}$$

The correlation coefficients vary from 0.256 ±0.116 (in the

TABLE 2

Logarithms of numbers of bacteria found in 1 cc. of peptone solutions after varying incubation periods

	AVERAGE (HOURS)														
	0	12	24	36	48	60	72	84	96	108	120	132	144	156	168
College Farm	4.78	6.40	7.01	7.43	8.11	7.96	7.98	7.97		7.86	7.88	7.81	7.98	7.34	7.34
College Farm, leached	4.48	6.59	6.99	7.42	7.52	7.84	7.91	8.12	7.70	8.15	8.20	8.09	8.12	7.96	7.60
NaCl, added leached	4.48	6.68	6.57	7.00	7.68	7.79	7.93	8.03	7.16	8.03	8.02	8.01	8.13	8.09	7.35
Na_2SO_4, added leached	4.11	7.17	7.26	7.86	7.96	8.20	8.32	8.50	8.45	8.48	8.39	8.27	8.17	8.16	8.15
Na_2CO_3, added leached	4.20	6.99	7.31	7.60	7.53	7.94	7.99	8.05	8.16	8.13	8.15	8.09	8.05	7.98	7.96
$NaCl—Na_2SO_4$, leached	4.11	6.66	6.91	7.70	7.70	8.23	8.35	8.48	8.59	8.53	8.21	7.81	8.56	8.34	8.43
$Na_2SO_4—Na_2CO_3$, leached	4.30	6.54	7.27	7.49	8.08	8.03	7.87	7.80	7.87	7.94	7.74	7.82	7.54	7.71	7.65
$Na_2SO_4—NaCl—Na_2CO_3$, added leached	4.18	6.54	7.38	7.77	8.02	8.07	8.28	8.04	7.80	7.86	7.95	8.07	7.98	8.04	7.98
Corinne, leached	4.42	6.52	6.94	7.26	7.73	7.94	8.12	8.17	8.03	8.23	8.35	8.50	8.48	8.42	8.18
Richland Acres, leached	3.78	6.48	7.16	7.71	7.79	7.91	7.90	8.11	7.85	7.78	7.91	7.93	7.89	7.86	7.83
Benson, leached	3.90	6.52	7.18	7.62	7.54	7.85	8.02	7.92	7.87	7.91	7.83	7.83	7.83	7.90	8.07

case of the sulfate-carbonate soil) to 0.870 ±0.044 in the case of the Corinne-leached soil, which in the case of the latter may be significant. However, the great fluctuation in numbers from period to period and the small number of determinations make it necessary that care be used in drawing final conclusions. It is evident, however, that alkali soil supplies a greater proportion of ammonifiers than does non-alkali soil. Even in the case of the alkali soil, a large proportion of the microörganisms which develop in peptone solution do not produce ammonia.

SUMMARY

The ammonifying powers of seven leached synthetic, three leached natural soils, and one native non-alkali soil were determined by inoculation in peptone solutions. Ammonia determinations as well as total counts were made every twelve hours for 168 hours. Ammonification data were fitted with satisfactory results to Pearl and Reed's formula:

$$y - d\frac{K}{1 + Ce^{rt}}$$

The process of ammonia accumulation by mixed cultures of soil bacteria grown in peptone solution closely approximates this equation.

The influence of the different salts on the ammonifying process of the soils studied is discussed.

The correlation between total numbers and ammonia accumulation varied from 0.426 ±0.159 in the College Farm soil to 0.870 ±0.116 in the Corinne-leached soil. A greater proportion of the microörganisms which will multiply in peptone and produce ammonia come from alkali soil than from non-alkali soil. A large proportion of the microörganisms which develop in peptone solution do not produce ammonia.

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A COMPARISON OF LEAD, BISMUTH, AND IRON AS DETECTORS OF HYDROGEN SULPHIDE PRODUCED BY BACTERIA

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Several methods have been used to detect hydrogen sulphide produced by bacteria. Filter papers impregnated with lead carbonate or lead acetate have been utilized ever since Gayon (1877) demonstrated the formation of hydrogen sulphide from albuminous material by bacterial action, and Schardinger (1894) applied the test to the sanitary examination of water. Sasaki and Otsuka (1912), and Tanner (1917) treated the lead acetate papers with glycerol. The addition of lead acetate or lead carbonate to the nutrient media has been especially advocated by Kligler (1917), Jordan and Victorson (1917), Thompson (1921), Bailey and Lacy (1927), and others. Wilson (1923) proposed the use of ferric chloride media to detect sulphides. After trying nickel, iron, lead, and manganese salts Levine and coworkers (1932) concluded that ferric citrate was one of the best indicators. Pacheco and Mello (1932) recommended bismuth carbonate agar which they found to be more sensitive and less toxic than iron or lead media.

Because difficulty has been encountered in testing a large number of recently isolated bacteria for their hydrogen sulphide producing properties as an aid in characterizing them for identification, and because the Committee on the Pure Culture Study of Bacteria (1933) is not ready to recommend any one method for such determination the following studies were undertaken.

EXPERIMENTAL

A comparison of different salts of bismuth, iron, lead, nickel, manganese, copper and tin showed that only the first three merited further consideration as indicators of small quantities of hydrogen sulphide in nutrient agar. The sensitivity of these minerals for detecting the presence of sulphide was tested in a medium consisting of 30 grams Bacto-peptone, 2.0 grams beef extract, 2.0 grams sodium chloride, 1.0 gram dibasic potassium phosphate, and 15 grams agar per liter. Just prior to the test various amounts of sodium sulphide were added to tubes of the molten media at 45°C. together with the mineral salts. The results were read after the media had cooled and solidified.

It was found that the smallest quantity of sulphide indicated by bismuth salts is 0.25 millimol sulphide S, 0.2 millimol by lead salts, and 0.1 millimol sulphide S by ferrous iron compounds. The minimum concentration of these cations required to produce a perceptible coloration in media containing 0.5 millimol sulphide S is 0.1 millimol ferrous iron, 0.2 millimol lead, or 0.5 millimol bismuth. Twice this concentration of each cation gives more satisfactory contrasts. Although, chemically, ferric iron is a poor sulphide indicator it is considered because, regardless of whether small quantities of ferric or ferrous iron are added to nutrient media, following heat sterilization and the growth of microörganisms both forms of iron will be present in concentrations which vary with the oxidation-reduction potential of the substrata.

The effect of these minerals on the multiplication of microörganisms was tested in the medium described above. After the addition of different quantities of iron, bismuth and lead salts, the reaction was adjusted to pH 7.2. The media were used to plate appropriate dilutions of sewage effluent. Table 1 shows the number of bacteria which developed from comparable quantities of sewage.

Not only was there a diminution in the number of colonies which developed on the media containing the higher concentrations of iron, lead, and bismuth but there was also a decided decrease in the size of the colonies which did appear. From the data of several experiments similar to those presented in table 1 it was found that, in general, concentrations of iron up to 4.0 millimols ferric iron or 2.0 millimols ferrous iron did not inhibit the multiplication of sewage bacteria. In fact, small quantities of iron stimulated bacterial growth. It made little difference whether the chloride, sulphate, citrate or acetate of iron was used, provided the H-ion concentration was properly adjusted.

As little as 0.2 millimol lead retarded the growth of some sewage bacteria (see table 2). Less than this quantity of lead was not tried because it had previously been demonstrated that at least 0.2 millimol was required to detect sulphides in peptone media. The toxicity of the lead was increased when the concentration of peptone was decreased. The presence of either glucose or lactose had no apparent effect on the bacteriostatic action of the lead. Equivalent quantitities of lead were equally toxic regardless of whether the carbonate, basic acetate or neutral acetate was used.

TABLE 1

Number of bacteria from a sample of diluted sewage which developed on media containing various concentrations of iron, lead, or bismuth

MILLIMOLS CATION	FERROUS CHLORIDE	FERRIC CHLORIDE	LEAD ACETATE	BISMUTH SUBCARBONATE
None	197	197	197	197
0.2	194	200	181	184
0.5	203	192	103	161
1.0	212	228	76	126
2.0	216	206	21	88
4.0	170	219	3	52
7.5	38	124	0	17
10.0	0	75	0	0

TABLE 2

Number of sewage bacteria developing on media containing varying quantities of lead acetate expressed as millimols lead

SEWAGE SAMPLE NUMBER	MILLIMOLS LEAD					
	None	0.2	0.5	1.0	2.0	4.0
14	236	206	158	103	32	2
22	304	272	196	114	26	8
23	43	34	19	9	0	0
26	128	83	74	41	5	2
27	260	198	164	122	19	0
28	87	70	53	46	11	3

Since bismuth salts are insoluble, media which contained either the subcarbonate or nitrate of this mineral were chalky, making it difficult to count the colonies which grew on such media. However, it was obvious that 1.0 millimol bismuth retarded colony development and that 4.0 millimols inhibited the growth of over half of the sewage bacteria. Similar effects were observed from the use of equi-molar quantities of bismuth as either the subcarbonate or nitrate in the lower concentrations, but the nitrate was more toxic when concentrations in excess of 4.0 millimols bismuth were present.

Next, the effect of iron, lead, and bismuth salts on the multiplication of several pure cultures was investigated. The latter consisted of 20 common bacteria including members of the colon-aerogenes group, spore-forming rods and cocci, all of which had been maintained in the laboratory for several years, and a similar number of very recently isolated marine bacteria. Media similar to those described above were poured into Petri dishes and the plates were divided into eight sectors with a wax pencil. The sectors were uniformly streaked with a standard loopful of the test organisms. Suspensions of the organisms were prepared by emulsifying a loopful of a twenty-four-hour-old agar slant culture in 5.0 cc. physiological saline solution. Thus, each medium was seeded with approximately the same number of cells. The inoculated plates were incubated at 30°C. and examined for comparative growth after twenty-four hours. Table 3 illustrates the kind of record which was kept.

To summarize briefly the results, it was found that the recently isolated bacteria were much more sensitive to lead, iron, and bismuth salts than were the old laboratory cultures. Although their growth was definitely retarded most of the former multiplied to some extent on 0.2 millimolar lead media. Some of the recently isolated cultures failed to grow on 0.5 millimolar lead media, less than half of them developed in the presence of 1.0 millimol lead even after four days, and none of these young strains tolerated 2.0 millimols lead. The growth of a few of the old stock cultures, lightly inoculated as in this experiment, was retarded by 0.2 millimolar lead media, but all of them multiplied after four days on 0.5 millimolar lead media. The majority of the old strains did not multiply in the presence of 2.0 millimols lead. These findings corroborate the results obtained from plating sewage bacteria and warrant the conclusion that while most bacteria will grow on media containing 0.5 millimol lead, the multiplication of many of them is abnormal. For comparison, 0.5 millimol lead is approximately equivalent to 0.02 per cent lead acetate [$Pb(CH_3COO)_2 \cdot 3H_2O$].

Concentrations of ferric or ferrous iron up to 2.0 millimols seemed to accelerate bacterial multiplication and all of the pure cultures tolerated 4.0 millimols of these cations. Most of the cultures grew in the presence of 7.5 millimols iron. The development of none of the pure cultures was inhibited by 1.0 millimol bismuth, but the growth of some was retarded by twice this amount.

TABLE 3

Comparative growth of bacteria after twenty-four hours' incubation on media containing varying quantities of lead as lead acetate

TEST ORGANISM	MILLIMOLS LEAD					
	None	0.2	0.5	1.0	2.0	4.0
Bact. paratyphosus (pigeon)	xxxx	xxxx	xx	x	—	—
Bact. aertrycke	xxxx	xxxx	xxxx	xx	x	—
Bact. coli-communis	xxxx	xxxx	xxxx	xxx	x	x
Strept. fecalis	xxxx	xx	xx	—	—	—
Staph. albus	xxxx	xxx	x	—	—	—
Sarcina lutea	xxxx	xxxx	xxxx	xx	x	—
Micr. tetragenus	xxxx	xxx	—	—	—	—
B. megatherium	xxxx	xxx	xx	x	—	—
Marine 501	xxxx	—	—	—	—	—
Marine 516	xxxx	xxx	x	—	—	—
Marine 534	xxxx	xx	x	—	—	—
Marine 568	xxxx	x	x	—	—	—

Although the addition of iron, lead, and bismuth salts to appropriate media have all been found to detect, with limitations, the hydrogen sulphide produced by some bacteria, such tests are not applicable to certain pigmented bacteria and others which normally darken the media because this coloration masks the color of the metallic sulphides which may be formed. Such microörganisms are not uncommon. Under these conditions the use of filter paper strips impregnated with the proper mineral salt is desirable. A comparison of different salts of manganese, tin, copper, iron, lead, bismuth, silver, mercury, and nickel, proved that while papers impregnated with some of these substances indicate the presence of hydrogen sulphide, lead acetate papers are superior, being more specific and more sensitive. In practice, strips of white filter paper measuring 5 by 50 mm. were soaked in a saturated solution of lead acetate, steam-sterilized in plugged tubes and dried in an oven at 120°C.

Sensitivity tests showed that lead acetate papers detect as little as 0.01 millimol sulphide S in peptone media. It will be recalled that at least 10 times this concentration of sulphide was required to darken perceptibly similar media containing the most efficacious mineral indicator, viz., ferrous iron. When much meat extract and other colored ingredients were present in the media, the lead acetate papers were found to be from 10 to 100 times (depending upon the coloration and reaction of the medium) more sensitive than iron, lead, or bismuth media for detecting traces of hydrogen sulphide. Treating lead acetate papers with glycerol to increase the hydroscopicity as practiced by some investigators has not been found to improve their usefulness. The importance of extreme sensitivity of the test for hydrogen sulphide production will be appreciated when one notes the small quantity of reduceable sulphur available in most peptones, and the minute amount of hydrogen sulphide produced by some bacteria, even from an adequate source. We have found Bacto-tryptone to be more suitable for the test than any other commercial peptone tried from a viewpoint of available sulphur content, growth-promoting properties, and uniformity of results.

CONCLUSIONS

There is a very narrow margin of safety between the concentration of lead salt in media which is required to detect sulphides and that which inhibits the multiplication of bacteria. While there is a vast difference in the lead-sensitivity of different bacteria, for those herein considered the ratio of the required concentration of lead to the average inhibitive concentration is 1:2. There is a larger margin of safety for certain lead-tolerant bacteria and particularly for old strains adapted to laboratory culture, but on the other hand, some lead-sensitive bacteria are inhibited by the least amount of lead acetate which is required to give a distinct color change with sulphides. In classifying 434 representatives of the Brucella group according to their ability to produce hydrogen sulphide ZoBell and Meyer (1932) found both lead acetate and lead carbonate to be entirely unsuitable as indicators because as little as 0.2 millimol lead inhibited the multiplication of some of these microörganisms. There was so much difference in the lead tolerance of different Brucellas that their cultivation on lead acetate agar became a differential test for their ability to tolerate lead rather than a test for their ability to produce hydrogen sulphide.

The average ratio of the required concentration of ferrous or ferric iron in nutrient media to the inhibitive concentration is about 1:40. Since the chalky appearance of bismuth media obscures the signs of bacterial multiplication, it is more difficult to estimate the ratio of the required to the inhibitive concentration, but it is about 1:5. Though comparative rather than absolute, these ratios indicate the superiority of iron salts as sulphide indicators. In practice we have found iron media much more satisfactory than either lead or bismuth. The use of 0.05 per cent ferric ammonium citrate or 0.03 per cent ferrous acetate is recommended in media containing at least 3.0 per cent peptone.

However, for testing new and unknown pure cultures of bacteria for their hydrogen-sulphide-producing properties as a means of characterizing them for identification and classification, the time-honored lead acetate paper method seems even better than ferrous iron media. Papers are more quantitative since the blackened area of the papers can be more readily estimated than the degree of darkening of iron media. Redfield (1912) states that "it is possible with a fair degree of accuracy to determine the equivalent of hydrogen sulphide for each millimeter of blackening of lead acetate paper strips." For recording the actual results the exposed lead acetate papers can be labeled, mounted in series and photographed or filed for future reference.

In advocating lead acetate papers, cognizance is taken of the wide application of the use of differential media containing lead, iron, or bismuth for such special tests as distinguishing between members of the typhoid-paratyphoid, colon-aerogenes or other special groups of bacteria. It is not our intention to criticize these tests because they have already proved their usefulness. Furthermore for studying the sulphur metabolism of microörganisms, there is no substitute for quantitative procedures such as those described by Almy and James (1926) and others.

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A NOTE REGARDING THE LAG PERIOD

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In a recent paper, Miss Ingraham (1933) presented data which imply a relationship apparently not recognized by the author, and which the writer, in his casual study of bacteriology, has never heard mentioned.

Miss Ingraham reported that crystal violet prolongs the lag period of bacteria but does not influence the rate of growth during the logarithmic phase. She also presented charts showing that, when the time required for cultures to become turbid was plotted against the logarithm of the inoculation, a straight line was obtained whose slope varied with the concentration of dye.

If these observations are correct, then it necessarily follows that, in the media used, *the length of the lag period is a linear function of the logarithm of the inoculation.* Of course, it is generally admitted that the lag period does vary according to the inoculation, but to the writer's knowledge, such a quantitative relationship has not been known.

This conclusion may be derived from the following considerations. First, the number of bacteria required to produce turbidity in a given medium should be a constant, independent of the inoculation. Second, if the growth rate in the logarithmic phase is constant, then the time required (excluding the lag period) should increase by a constant amount (the generation time) each time that the inoculation is reduced by half. Then, if the lag period were a constant, C, independent of the size of the inoculation, the following relation should hold:

$$T_t = K \log \frac{N_t}{N_i} + C \qquad (1)$$

where T_t = the time required for turbidity, C = the lag period, a constant, K = a constant, the time required for the bacteria to multiply tenfold, N_t = the bacterial count when turbidity appears, and N_i = the bacterial count at inoculation.

This equation gives the relation between the time required for turbidity and the size of the inoculation. It represents a straight line whose slope, K, depends only upon the rate of multiplication.

Miss Ingraham reported that the generation time, and consequently K, was not changed by crystal violet. Nevertheless, she found that the slope of the line was changed by the presence of dye. If K is actually a constant, then this fact can only be explained by asserting that the lag period is not constant, but actually changes with the size of the inoculation.

This conclusion makes it necessary to rewrite the above equation replacing the constant, C, by a variable term expressing the relation between the length of the lag period and the size of the inoculation.

The nature of this term may be deduced from the following considerations. First T_t has been found, experimentally, to be a linear function of the logarithm of the inoculation. Second, T_t is the sum of two terms, the first representing the growth period and the second, the lag period. But the length of the growth period is a linear function of the logarithm of the inoculation. This necessitates that the lag period must, also, be a linear function of the logarithm of the inoculation, as expressed by the following equation:

$$T_L = C_1 - K_2 \log N_i \tag{2}$$

where T_l = the length of the lag period, C_1 and K_2 are constants, and N_i = bacteria count after inoculation.

The changing slope observed by Miss Ingraham can be accounted for by variation of K_2 in the presence of dye.

Equation 2 is the relationship which was mentioned as a necessary corollary of Miss Ingraham's results. If this relationship is found to be generally applicable, it may lead to an increased knowledge of the true nature of the lag phase.

The complete equation for the relation between the inoculation and the time required for turbidity would be:

$$T_t = K \log \frac{N_t}{N_i} + C_1 - K_2 \log N_i$$

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ISOLATION OF AN OBLIGATELY ANAEROBIC BACILLUS FROM THE FECES OF NEWBORN INFANTS AND FROM OTHER HUMAN SOURCES, AND ITS PROBABLE IDENTITY WITH THE "KÖPFCHENBAKTERIEN" OF ESCHERICH, RODELLA'S "BACILLUS III," AND BACILLUS PARAPUTRIFICUS (BIENSTOCK)

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During a preceding study of the intestinal flora of the first ten days in newborn infants, Hall and O'Toole (1934) isolated from six babies out of ten, 18 strains of obligately anaerobic bacilli with oval terminal spores which were tentatively identified as the "*Köpfchenbakterien*" seen by Escherich (1885) (1886) in the feces of newborn infants. A more detailed study of six of these, one from each baby, together with fourteen additional strains which we have isolated from other human materials, showed them to belong to a single species which we regard as the *Bacillus paraputrificus* of Bienstock. Although the probable identity of this species with the "*Köpfchenbakterien*," "*Köpfchensporen*," and "*Köpfchenbacillen*" of the older German writers was clearly established by Bienstock in 1906, modern French, English, and American bacteriologists have generally omitted any accurate description of this bacillus, while modern German writers and a few others have continued to use the term "*Köpfchenbakterien*" with reference either to a species or group of species of anaerobic bacilli with oval terminal spores found in the feces of infants.

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We are indebted to Miss Elisabeth O'Toole for her excellent flagellar stains and to Mr. Glen Mills for the photomicrographs.

HISTORICAL

Bienstock (1884) was probably the first to discover a bacillus with round or oval terminal spores in cultures from feces. These cultures were aerobic and Bienstock referred to the organisms in them as "*Köpfchensporen*," "*Trommelschlägerform bildenen Bazillus*," and "*Bazillus der Eiweissfäulniss*." Bienstock is sometimes cited as having used the specific name *Bacillus putrificus-coli* in 1884 but our examination of his original paper failed to verify this.

Escherich (1885, 1886) described "*Köpfchenbakterien*" in films of feces and in mixed anaerobic cultures as slender rods, 4 to 7μ long, with bright glistening terminal spores which made them resemble spermatozoa, and clearly recognized their probable identity with Bienstock's "*Eiweissfäulniss bacillus*." Subsequent studies have shown that the "*Köpfchenbakterien*" are obligately anaerobic, but, in spite of the fact that Escherich secured anaerobic cultures under vacuum from the feces of newborn infants, there is nothing to indicate that he ever isolated the "*Köpfchenbakterien*" in pure culture.

For this reason, it is impossible with certainty to identify with the "*Köpfchenbakterien*" any of the microörganisms isolated from infant stools either by ourselves or others, but the high frequency of a single species with the morphology of the "*Köpfchenbakterien*" in the feces of newborn infants is strong presumptive evidence of identity.

Owing to the important significance of the early recognition by both Bienstock and Escherich of bacilli with oval or nearly round spores in the feces, and the absence of their monographs from all but a few libraries in the United States, photographic copies of their illustrations are here reproduced as figures 1 and 2.

Curiously there is no reference to Escherich's "*Köpfchenbakterien*" in the studies on intestinal flora of nursing infants by Gessner (1889), Popoff (1891), Schmidt (1892), Schild (1895), Eberle (1896), Szego (1897), Moro (1900), and Cahn (1901) during a decade and a half following Escherich's first observations. It is equally impossible to identify with the "*Köpfchenbakterien*" any of the oval or round terminally-sporulating anae-

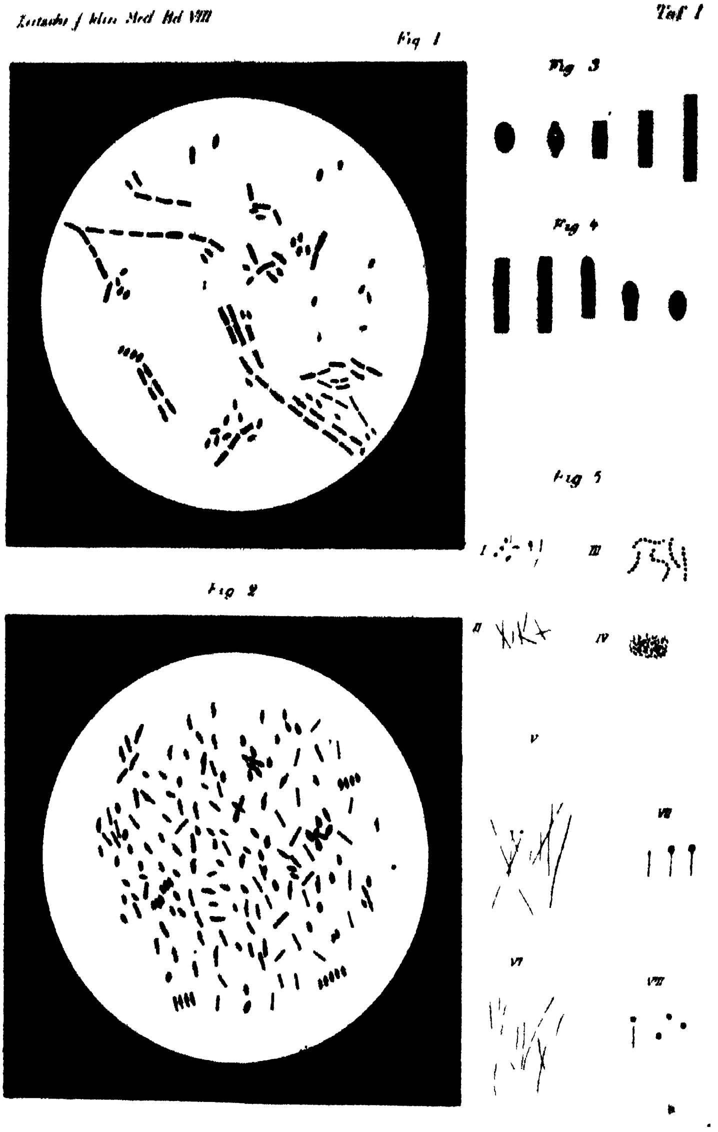

FIG. 1 PHOTOGRAPHIC REPRODUCTION OF BIENSTOCK'S PLATE I, SHOWING VARIOUS BACTERIA SEEN IN FECAL CULTURES

These were apparently drawn in colors diagrammatically, and probably on different scales, from slides stained with warm aniline fuchsin, decolorized in 33⅓ per cent nitric acid and counter-stained with methylene blue according to the methods of Neisser and Ehrlich Figure 7 represents the supposed "Bazillus der Eiweissfaulniss," whose actual identity is now only conjectural robic bacilli described by Liborius (1886), Gruber (1887), Lüderitz (1889), Sanfelice (1893), or Tavel (1898) owing to their

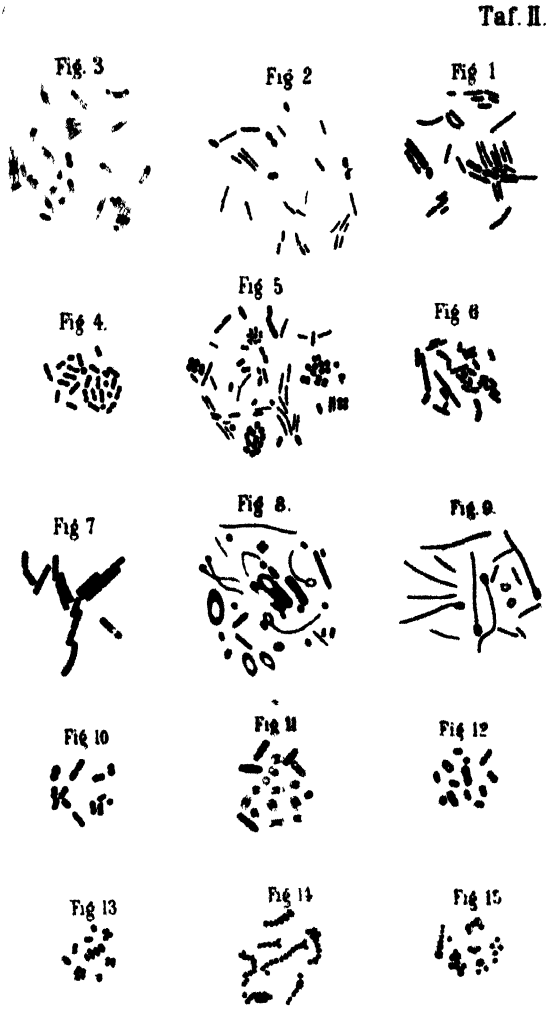

Fig. 2. Photographic Reproduction of Escherich's Plate II, Showing Various Bacteria Seen in Meconium and in Fecal Cultures

The "Kopfchenbakterien" ("Bienstock's Eiweissbazillus") are shown in figures 8 and 9.

inadequate description for modern identification. On the other hand *"Köpfchenbakterien"* have been isolated since 1898 by various investigators.

In 1899, Klein recovered obligately anaerobic non-pathogenic "*Trommelschlägerformen Köpfchenbacillen*" from buried bodies which he sharply distinguished from Bienstock's aerobic "*Bacillus putrificus coli*" under the name "*Bacillus cadaveris sporogenes.*"

In the same year, Bienstock (1899) attempted unsuccessfully again to recover from feces a proteolytic aerobe similar to his original "*fäulnisserregender Bazillus.*" Failing this, he succeeded in isolating from street manure obligately anaerobic "*Köpfchensporen*" which he regarded as identical with the "*Eiweissfäulniss Bazillus,*" taking the view that his earlier culture was a contaminated symbiotic mixture of this organism with aerobic forms. In the following year (1900) Bienstock admitted that his original fibrin medium in 1884 was insufficiently sterilized, that *B. putrificus* rarely if ever occurred in feces, and that it was probably identical with Klein's *Bacillus cadaveris sporogenes.* This viewpoint was accepted by von Hibler (1908).

In these papers, Bienstock emphasized the peculiar morphology of *B. putrificus,* its liquefying action upon proteins, the failure of various carbohydrates to protect the proteins against decomposition by pure cultures, and the inhibitive action of *B. coli* upon proteolytic changes in milk when grown with *B. putrificus.*

Subsequently, Achalme (1902) recorded the fermentation of several carbohydrates by cultures of "*B. putrificus*" secured from the Pasteur Institute in Paris, but Tissier and Martelly (1902), and later Rodella (1905), pointed out that the Paris cultures were contaminated by *Bacillus bifermentans sporogenes* and that pure cultures of *B. putrificus* did not ferment the sugars. Their failure to do so was re-iterated by Bienstock in 1906.

This point is important because it lays the basis for the later identification of *B. putrificus* by Reddish and Rettger (1922) (1923) (1924), Hall (1922), Kahn (1922), Cunningham (1932), and others, and our recognition of the "*Köpfchenbakterien*" as belonging to a single separate species which ferments several sugars and has neither gelatinolytic nor proteolytic action. For the same reason we are unable to accept Sittler's (1909) and Cruickshank's (1931) identification of "*Köpfchenbakterien*" with *Bacillus putrificus,* or to understand the inclusion in the species

B putrificus, of various non-pathogenic anaerobes with oval terminal spores, by Weinburg, Aznar, and Duthie (1924) irrespective of fermentation reactions. Niszle's (1929) recent summary clearly shows the generally confused state of present knowledge concerning these microörganisms.

The first confirmation of "*Köpfchenbakterien*" in the feces of newborn infants after Escherich appears to have been made by Tissier (1900), who, however, made the common mistake of regarding them as "attenuated" strains of *B. putrificus* until Rodella (1902) pointed out their lack of proteolytic properties, a criticism which Tissier graciously acknowledged in 1905 and again in 1908. Rodella's work with infants' stools began in 1901 in a study of the acidophilic bacteria which had just been so carefully described by Tissier (1900) and Moro (1900). In the following year Rodella (1902) described three numbered but unnamed species of obligately anaerobic gas-forming bacilli from the normal stools of nursing infants. Rodella regarded No. III as identical with Escherich's "*Köpfchenbakterien*," and described it in pure culture as forming slender Gram-positive rods with round terminal spores, not liquefying gelatin, or changing milk, producing abundant gas in broth, and being non-pathogenic for laboratory animals.

Rodella's Bacillus III was again found by Tissier (1905) in the lower bowels of newborn infants during the early stage of intestinal invasion by bacteria, in the stomachs and both upper and lower bowels of puppies, and later (1908) in the feces of infants one to five years old.

Rodella's observations on Bacillus III and its identity with Escherich's "*Köpfchenbakterien*" were also confirmed by Moro (1905). This writer recorded the occasional coagulation of milk and the motility of the vegetative rods which Rodella had failed to see. Moro erred, however, in regarding the "*Köpfchenbakterien*" as sporulating forms of *Bacillus* (*Lactobacillus*) *bifidus*.

Passini (1905) likewise studied several strains of anaerobic bacilli among which were some identified as Escherich's "*Köpfchen-Putrificuskeim.*" Bienstock (1906) regarded these as corresponding to his "*Bacillus paraputrificus*" which, unlike his *B.*

putrificus, often coagulated milk, forming an acid whey without subsequent liquefaction of the casein, and fermented both glucose and lactose. It seems entirely probable that Bienstock's *Bacillus paraputrificus* was identical both with the *Köpfchenbakterien* of Escherich and with Rodella's Bacillus No. III. Although the name *Bacillus paraputrificus* has apparently not since been connected in the literature with either of these microörganisms, we are quite convinced of its validity and shall henceforth use it for the species under discussion.

In 1908 von Hibler described as Bazillus IX, a non-pathogenic obligately anaerobic rod isolated ten years before from the emphysematous tissues of a boy having a compound fracture of the humerus. Von Hibler considered this culture similar to *Bacillus solidus* of Lüderitz, but its properties clearly identify it with Rodella's Bacillus III, as suggested by Robertson (1916).

Fleming (1915) and Goadby (1917) had also observed terminally spored bacilli in war wound infections, and Robertson regarded these and certain morphologically similar cultures, which she isolated, as also identical with Rodella's Bacillus III and von Hibler's Bacillus IX. These cultures were subsequently named *Bacillus tertius* by Henry (1917), thus starting a new train of confusion which has been perpetuated by Weinberg and Sequin (1917), McIntosh (1917), Bullock (1919), Douglas, Fleming and Colebrook (1920) and Robertson (1929). For, in spite of their superficial resemblance, *Bacillus paraputrificus* (Bienstock) is clearly distinct from *Bacillus tertius* (Henry), as the following comparison shows.

	Bacillus paraputrificus	*Bacillus tertius*
Aerobic growth	–	+*
Morphology of spores	Oval	Elongate
Fermentation of xylose	–	+
Fermentation of mannitol	–	+

* See Hall and Matsumura (1924).

In short, von Hibler's Bazillus IX was probably identical with Bienstock's *Bacillus paraputrificus* and Rodella's Bacillus III, but *Bacillus tertius* is a distinct species. This conclusion suggests that the English observations cannot be accepted as evidence of the occurrence of Rodella's Bacillus III or von Hibler's Bacillus IX in wound infections; on the other hand, the only valid evidence we have of its occurrence in wound infections rests upon von Hibler's limited observations and our own.

We believe that the only significance attached to the occurrence of *B. paraputrificus* in wound infections is the implication which it carries of fecal contamination. On the other hand, while *B. paraputrificus* frequently attains a moderate development in the intestinal tracts of newborn infants during the first week of life, the acidity of the normal meconium (pH 6.0 to 6.6) is by no means optimal; Adam (1921, 1922) has shown that the "*Köpfchenbakterien*" grow best in alkaline media (pH 6.9 to 8.2), and they disappear more or less completely upon the establishment of the normal milk stool because of the higher acidities which definitely favor *Lactobacillus bifidus* (optimum pH 5.0 to 5.8). However, *B. paraputrificus* may frequently be recovered from older children and from adults.

In view of the preceding studies in Germany, it is remarkable that Zeissler and Käckell (1922), in a study of 32 specimens of feces from 14 healthy and 11 ill babies, failed either to discuss or to isolate the "*Köpfchenbakterien*," and still more so that Schüssler (1924) in a special study recovered them only 4 times from 112 specimens of meconium from 48 newborn infants, and that he interpreted them as "degenerationsformen des *Bacillus amylobacter*." In this country Upton (1929) claims to have seen the "*Köpfchenbacillus*" only once during a study of the anaerobic intestinal flora of 89 specimens of feces from newborn infants (one to six days), but there is no indication that she succeeded in isolating it in pure culture.

SOURCES OF BACILLUS PARAPUTRIFICUS

All of our strains of *Bacillus paraputrificus* were isolated in our own laboratories during the past ten years. The first was recovered in 1924 from the feces of a normal child ten years old, and others were isolated at irregular intervals from materials secured from human autopsies and from various infections, but it was not until we began our systematic study of the feces of newborn infants in 1930 that we connected these organisms with Escherich's *"Köpfchenbakterien."* The twenty strains studied in detail were derived as follows:

	Number of strains
Feces of newborn infants	7
Feces of ten-year-old child	1
Fecally contaminated surgical wound (colostomy)	1
Decubitus ulcer on buttock	1
Gaseous gangrene (amputation of leg)	1
Postmortem heart blood cultures	5
Postmortem peritoneal fluid cultures	3
Postmortem pleural fluid cultures	1
Total	20

Analysis of our data shows that in all cases other intestinal bacteria were associated, as follows:

	Number of strains
Bacterium coli	14
Streptococcus species	9
Micrococcus species	6
Bacterium aerogenes	6
Bacillus Welchii	5
Bacillus tertius	3
*Bacillus difficilis**	2
Bacterium alkaligenes	2
Bacillus centrosporogenes	2
Lactobacillus species	2
Bacillus Sordellii	1
Bacillus botulinus	1
Bacterium proteus	1
Bacterium pyocyaneum	1
Bacterium flavescens	1

* *Bacillus difficilis* was recently described as a new species of pathogenic anaerobe by Hall and O'Toole (1934).

ISOLATION OF PURE CULTURES

It will readily be understood that isolation of pure cultures of *B. paraputrificus* was by no means an easy task, although less difficulty was encountered than in the case of *B. difficilis,* and when these two anaerobes occurred together in the feces of infants, *B. paraputrificus* was always recovered first in pure culture. On the other hand, when other bacilli were associated they were generally isolated before *B. paraputrificus.*

In general, the methods used were those advocated by Hall (1932). Primary mixed cultures in deep brain medium were transferred to blood agar slants, made anaerobic by means of alkaline pyrogallol and incubated at 37°C. for forty-eight hours. These cultures were placed in a bath of boiling water for two minutes and the melted agar transferred by pipette to tubes of deep brain medium similarly heated to drive out dissolved oxygen. When anaerobic bacilli were present, incubation of these resulted in gas production. Some of these cultures proved to be pure *B. paraputrificus;* several contained other sporulating anaerobes. In any event, dilutions were streaked out upon blood agar slants which were then made anaerobic with alkaline pyrogallol and incubated, or dilutions were made in deep agar tubes; from either of these, well separated colonies were picked into deep brain medium. These procedures often had to be repeated or alternated several times before pure cultures were secured.

MORPHOLOGY

One occasionally sees in smears of feces of infants, or of other sources, sporulating rods resembling spermatozoa. These may or may not prove to be *Bacillus paraputrificus.* In cultures vegetative cells appeared as Gram-variable slender rods with rounded ends and measuring 2 to 6μ in length and 0.3 to 0.5μ in width. The rods were straight or slightly curved and occurred singly, in pairs, and sometimes in short chains.

Spores developed readily in brain medium and blood agar slants under alkaline pyrogallol, and were seen in all stages. Young spores (orgonts) stained solidly by Gram's method, but the mature oval spores were refractory to staining; they measured 1.2μ in length, 1.0μ in width. Figure 3 shows orgonts, spores, and vegetative rods as they appeared in a three-day-old blood agar slant.

MOTILITY

All of the strains were found to be motile in glucose broth and brain media, but the majority of the rods were non-motile. This observation is perhaps significant because Rodella failed to observe motility, but this is the only discrepancy between his observations and ours.

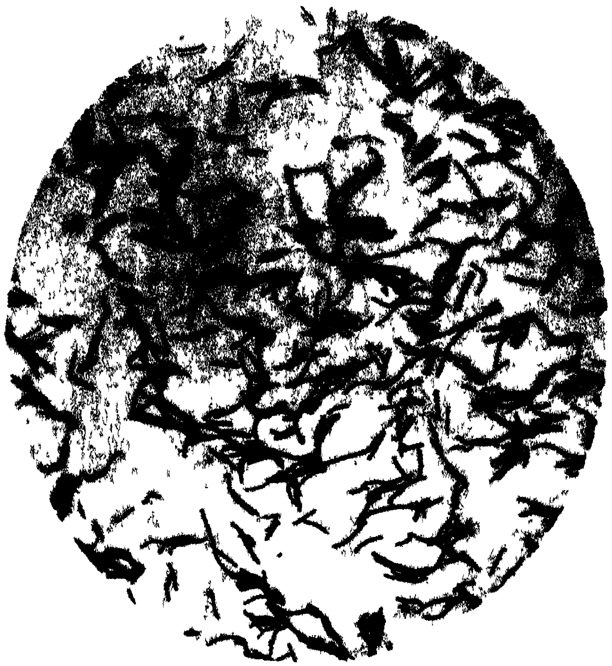

FIG. 3. B. PARAPUTRIFICUS (BIENSTOCK), FROM THREE-DAY-OLD BLOOD AGAR SLANT, STAINED BY GRAM'S METHOD TO SHOW VEGETATIVE RODS, ORGONTS, AND SPORES. × 1000

Flagella were stained by Bailey's method (1930); they were peritrichic and numbered 6 to 15, as shown in figure 4.

Sporulation generally resulted in loss of motility, but in one instance an orgont was stained with three flagella still attached, as shown in figure 5.

CULTURAL PROPERTIES

Deep 1 per cent agar. Colonies appeared in twenty-four to forty-eight hours as small irregular, opaque, dense, cottony masses rarely exceeding 1 mm. in diameter. They are illustrated in figures 6 and 7. Even with moderate seeding, considerable gas was generally produced.

Blood agar slants under alkaline pyrogallol. All strains produced delicate, irregular, non-hemolytic, round-topped colonies resembling dewdrops in young culture; older colonies were flat-topped.

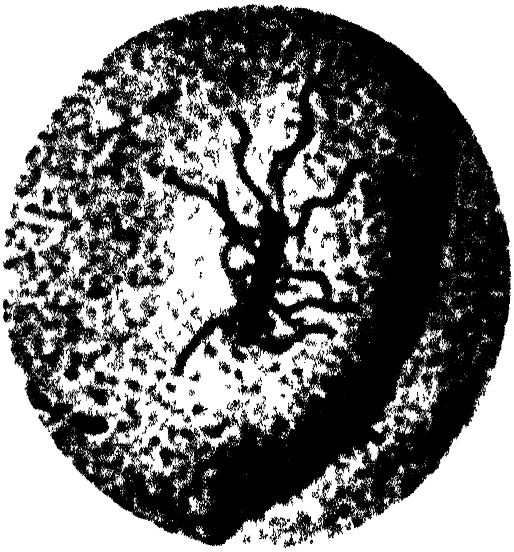

FIG. 4. BACILLUS PARAPUTRIFICUS (BIENSTOCK) FROM THREE-DAY-OLD BLOOD AGAR SLANT SHOWING PERITRICHAL FLAGELLA

Stained by Bailey's method About 15 flagella can be counted × 3300

Deep brain. Abundant gas was produced overnight. No blackening occurred on prolonged incubation.

Deep brain with iron wire. The results were the same as without iron during the first ten days. After that a moderate gray discoloration appeared at the top of the medium and along the upper part of the wire. Cultures capped with tin foil under cellophane showed after five months a brown deposit where the iron touched the glass, probably iron oxide; the surface of brain substance was covered with a gray deposit, but there was no blackening in the depths of this medium such as one sees in the case of putrefactive anaerobes where abundant H_2S is produced. Special tests of brain medium with 0.1 per cent lead acetate failed to show any blackening whatever, but we suspect nevertheless from the appearance of old iron brain cultures that a small amount of H_2S is formed.

There was little or no liquefaction of the brain substance; the microorganism is essentially non-proteolytic.

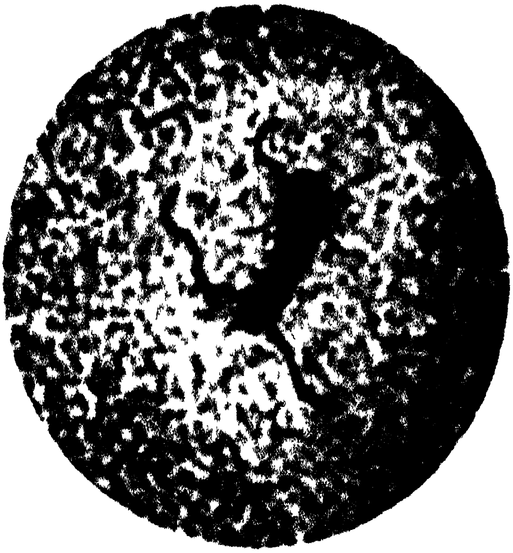

FIG. 5. BACILLUS PARAPUTRIFICUS (BIENSTOCK) WITH THREE FLAGELLA STILL ATTACHED TO A ROD UNDERGOING SPOROGENESIS

Stained by Bailey's method. × 3300

Loffler's coagulated blood serum. No liquefaction or discoloration occurred in thirty days.

Gelatin. Turbidity and a small amount of gas appeared under both vaspar and marble seals in constricted tubes in twenty-four hours. The turbidity disappeared in a few days, due to settling. No liquefaction occurred during twenty days observation.

Ten per cent powdered milk solution. Gas was produced both under marble seals in constricted tubes and under vaspar seals. One strain slowly coagulated the casein, but the remaining strains showed neither coagulation nor liquefaction in ten days.

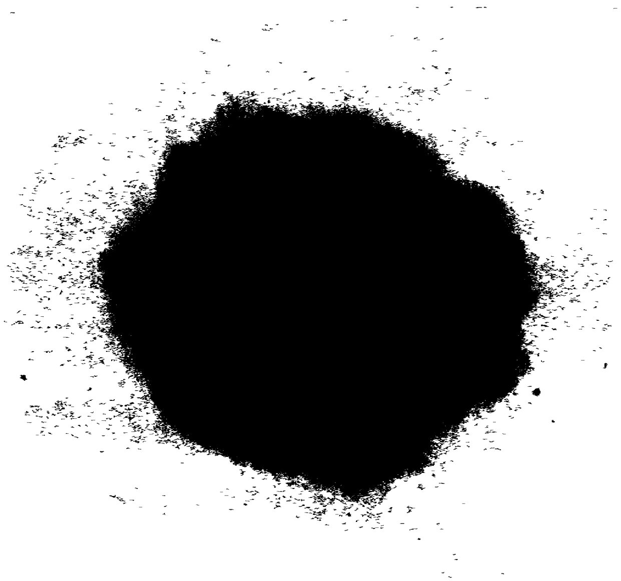

Fig 6

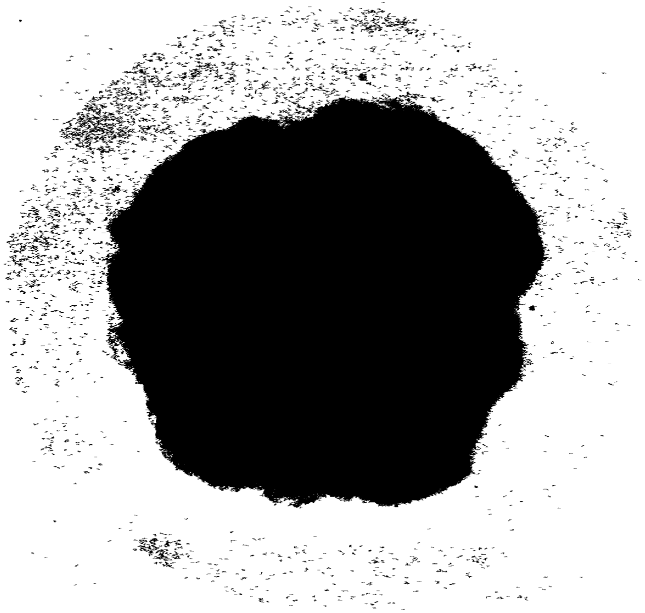

Fig 7

Figs 6 and 7 Three-day-old Colonies of Two Different Strains of Bacillus paraputrificus in Deep 1 Per Cent Agar (× 100), Indicating the Extremes of Variation in Density and Textures of Such Colonies

Sterile cow's milk. Abundant gas production was followed by slow coagulation in all but two strains in six to ten days. There was no liquefaction of the coagulated casein.

Difco proteose-peptone broth for indol. Turbid forty-eight hour cultures in this medium under marble seals in constricted tubes were shaken with ether and tested for indol by adding Ehrlich's reagent (paradimethylaminobenzaldehyde) to the ether layer. This test gave negative results with all of the strains. Control tests with *Bacillus tetani* gave positive results.

Fermentation reactions. These reactions were tested by two methods, as follows:

(*a*) Hiss serum water media containing 1 per cent of the various carbohydrates were prepared by filling slender tubes (150 mm. by 10 mm.) half full. Litmus was used as an indicator. The tubes were boiled for a few minutes to expel air, cooled, and inoculated with a few drops of fresh glucose broth cultures of the various strains. Glucose, levulose, galactose, maltose, lactose, sucrose, raffinose, dextrin, soluble starch, amygdalin, and salicin were fermented, as was shown by coagulation of the serum and by reddening and reduction of the litmus. No liquefaction of the coagulated serum occurred. Xylose, inulin, mannitol, and glycerol were not fermented.

We have used this method successfully for several actively fermentative anaerobes other than *B. paraputrificus*, but it is not recommended for general use; it failed entirely with the putrefactive anaerobes.

(*b*) The tests were repeated in sugar-free broth in constricted tubes with marble seals to which, after boiling to expel oxygen, sufficient 10 per cent sterile carbohydrate solutions were added to yield 1 per cent concentrations. The same sugars were fermented as before, as indicated by abundant gas production, and acid reaction to brom-thymol-blue upon removal to a spot plate.

Pathogenicity. Each strain was tested in guinea pigs by subcutaneous injection of 2 cc. of forty-eight-hour glucose broth cultures. No symptoms or lesions were observed.

Several of the strains were likewise repeatedly injected intravenously into rabbits in the preparation of agglutinating serums, without any evidence of pathogenicity.

SUMMARY

We have recovered from the feces of seven newborn infants, from the feces of a ten-year-old child, from a fecally contaminated surgical (colostomy) wound, from a decubitus ulcer, from five postmortem heart blood cultures, from three postmortem peritoneal fluids, from one post-mortem pleural fluid, and from the amputated stump of a gangrenous leg, an obligately anaerobic bacillus which appears to be identical with the *Bacillus paraputrificus* of Bienstock.

A study of the literature indicates the identity of this organism with Rodella's Bazillus III, von Hibler's Bazillus IX, and Escherich's "*Köpfchenbakterien.*"

The improper confusion of Escherich's "Köpfchenbakterien" with *Bacillus putrificus* (Bienstock), and of Rodella's Bacillus III and von Hibler's Bazillus IX with *Bacillus tertius* (Henry) has been traced in the literature, and the true distinctions between *Bacillus putrificus* (Bienstock), *B. paraputrificus* (Bienstock), and *Bacillus tertius* (Henry) have been pointed out.

Bacillus paraputrificus (Bienstock) is a motile, Gram-variable, slender, obligately anaerobic bacillus, with oval terminal spores, which forms small opaque cottony colonies in deep agar, delicate irregular non-hemolytic surface colonies on blood agar, abundant gas in brain and other liquid or semisolid media, without evidence of H_2S or indol production. It is non-proteolytic, non-gelatinolytic, and produces gas and often coagulum, but no liquefaction of casein in milk. It ferments glucose, levulose, galactose, maltose, lactose, sucrose, raffinose, dextrin, soluble starch, amygdalin, and salicin, with production of acid and gas; it fails to ferment xylose, inulin, mannitol, and glycerol, and is non-pathogenic for guinea pigs and rabbits.

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A COMPARATIVE STUDY OF CERTAIN MEDIA USED IN PRESUMPTIVE TESTS FOR BACT. COLI[1]

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In recent years the validity of standard lactose broth as a suitable presumptive test medium in sanitary water analysis has been seriously questioned because of several well recognized facts. First, *Bact. aerogenes*, considered to be of non-fecal origin, gives positive presumptive tests in lactose broth; second, various spore-forming bacteria also give spurious presumptive tests in this medium; and third, certain synergistic relationships between microörganisms in latose broth also yield spurious presumptive tests. Since the differentiation between safe and unsafe water supplies used for drinking purposes is of utmost importance for public health protection, and since the bacteriological examination revolves primarily around the detection of *Bact. coli* of fecal origin, it is not surprising to find that the bacteriological literature has abounded with contributions on this subject even in recent years.

In order to eliminate false-positive presumptive tests and to favor the development of *Bact. coli* over *Bact. aerogenes*, various new media have been recommended. Among these are crystal-violet lactose broth recommended by Salle (1929; 1930); the simultaneous use of lactose broth and brilliant-green lactose peptone bile recommended by Jordan (1932); the use of the Dominick and Lauter broth medium (1929); and the use of Tonney and Noble's ferrocyanide citrate agar (1931). While Leahy (1930) has supported the use of the Dominick and Lauter medium, Howard (1932) found it less desirable than brilliant-green lactose peptone bile. Stark and England (1933) disapproved of the use of Salle's medium and Butterfield (1933), comparing lactose broth and brilliant-green lactose peptone bile favored the use of the former as a more delicate instrument for detecting *Bact. coli*. Recently (1933), Parr and Caldwell (1933) concluded that "the use of green bile and lactose in parallel requires further trial before it can be generally adopted."

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[1] Contribution No. 32 from the Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Mass.

In the light of these various recommended substitutions for lactose broth as a presumptive test medium and the conflicting results obtained by various workers, it was considered worth while to make a comparative study of these media. To this end 50 different waters, mostly unfinished or unpurified, were examined, as well as freshly isolated cultures of *Bact. coli* from human, bovine and equine fecal material. Pure cultures of *Bact. aerogenes* were also studied. The media used for comparison included standard lactose broth, Salle's crystal-violet lactose broth, Dominick and Lauter's lactose broth, and Jordan's brilliant-green lactose peptone bile. Preliminary work with Tonney and Noble's ferrocyanide citrate agar using pure cultures of freshly isolated fecal strains of *Bact. coli* and stock cultures of *Bact. aerogenes* gave such unsatisfactory results in our hands that it was not included in the comparative study of the various waters.

PROCEDURE

For each water examined, parallel inoculations were made in the four fluid media enumerated above, using 10-, 1-, and 0.1-cc. amounts. Ten tubes of each medium were inoculated with each amount of inoculum, making 30 inoculations for each medium and 120 inoculations for the four media under comparison. The 0.1-cc. inoculation was made by using 1-cc. amounts of a 1:10 dilution in sterile distilled water. After inoculation, the media were incubated at 37°C. and observed at the end of twenty-four and forty-eight hours. Each tube showing a positive or doubtful presumptive test was examined further on eosin methylene-blue agar for the partially confirmed test. In positive cases, the inoculations were made from typical *Bact. coli* colonies into lactose broth and on to agar slants. A positive test in lactose broth and the appearance of Gram-negative, non-spore forming bacilli resembling *Bact. coli*, were the standards used for the completed test. The Kopeloff and Beerman Gram stain was employed for determining the Gram reaction of the organisms.

COMPOSITION OF MEDIA EMPLOYED

The culture media employed were prepared in every case but two from the dehydrated products of the Digestive Ferments Company. The exceptions were Salle's crystal-violet lactose broth which was prepared according to the formula given by the author (Salle, 1930) and the Dominick and Lauter medium prepared as described below. For lactose broth, Salle's medium and the 2 per cent brilliant-green lactose peptone bile, the culture media were distributed in Durham fermentation tubes in 10-cc. amounts when 1- and 0.1-cc. amounts of water were inoculated. When 10-cc. amounts of water were used for inoculation, large fermentation tubes containing 20 cc. of each medium were employed. For inoculations into Dominick and Lauter's medium, 15 cc. of the culture medium were employed regardless of the amount of test water inoculated. No changes were made in the composition of lactose broth regardless of the amount of water inoculated, but for the other media under comparison, variations in concentration were made in accord with the amount of water examined in order to maintain the proper ratio of inhibitory dye.

Salle's medium was prepared by dissolving in 2000 cc. the following ingredients: 15 grams of Difco peptone, 15 grams of K_2HPO_4, 3 grams of KH_2PO_4, 15 grams of Difco lactose and 21.5 cc. of a 1:5000 solution of crystal violet. This medium was distributed in fermentation tubes in 20-cc. amounts, sterilized in streaming steam for ten minutes and subsequently used for the 10-cc. inoculations of the test water. For the inoculations of the 1- and 0.1-cc. amounts of water the same ingredients were dissolved in 2727 cc. of distilled water, the medium being distributed in fermentation tubes in 10-cc. amounts.

The formula for brilliant-green lactose peptone bile requires 20 grams of oxgall, 10 grams of peptone, 10 grams of lactose and 0.0133 gram of brilliant green. These ingredients are dissolved in a liter of distilled water, distributed in the amounts desired and sterilized. For inoculating 1- and 0.1-cc. amounts of water, 40 grams of the dehydrated product per liter were employed, while for inoculating 10-cc. amounts of water, 60 grams of the dehydrated medium per liter were used.

The formula for the Dominick-Lauter medium was obtained at the Washington meeting of the American Public Health Association on October 27, 1932, and was reported as being the latest and most approved modification. It calls for 7.5 grams of Difco lactose, 12.5 grams of Difco peptone, 1.25 grams of Difco beef extract, 3.6 grams of K_2HPO_4, 0.5 gram of KH_2PO_4, 0.01 gram erythrosin, 0.016 gram of brom cresol purple (National Anilin Company) and 0.025 gram of methylene blue. These ingredients are dissolved in 1 liter of distilled water, distributed in 15-cc. amounts and sterilized. In this concentration, 10-cc. amounts of test water are inoculated. For 1- and 0.1-cc. inoculations, 10 grams of the above ingredients are dissolved per liter instead of the 25.4 grams per liter used for the 10-cc. inoculations. The weaker medium is also distributed in fermentation tubes in 15-cc. amounts and sterilized at 15 pounds pressure for fifteen minutes before use.

NUMBER OF ORGANISMS REQUIRED FOR POSITIVE PRESUMPTIVE TEST

One way of ascertaining the relative sensitiveness of the various presumptive test media under comparison to the presence of *Bact. coli* or *Bact. aerogenes* is to prepare bacterial suspensions of each organism and to make inoculations into the various test media after suitable dilutions. For this purpose, a twenty-four-hour agar slant culture of *Bact. coli* freshly isolated from human feces was employed on four different occasions, while a twenty-four-hour agar slant culture of a laboratory strain of *Bact. aerogenes* was used. The agar slant culture was washed off with 10 cc. of sterile distilled water and then introduced into a flask containing 290 cc. of sterile distilled water. After thorough shaking in order to insure uniformity, dilutions of 1:10,000, 1:100,000, 1:1,000,000 etc., were prepared from this suspension. Each dilution was examined for organisms capable of producing gas in each of the presumptive test media. For this purpose, 10 tubes of each medium were inoculated with 1-cc. portions of each dilution.

The cultures were incubated at 37°C. and observed at the end of twenty-four and forty-eight hours. Direct counts of the concentration of organisms in each dilution were made on nutrient agar at 37°C. after twenty-four hours.

From table 1 it is evident that standard lactose broth is the most sensitive presumptive test medium when freshly isolated *Bact. coli* is present as a pure culture in sterile distilled water. The same holds true for *Bact. aerogenes*, prepared in the same way. The second most sensitive presumptive test medium under the conditions of the experiment was brilliant-green lactose peptone bile; the third was Dominick and Lauter's medium and the fourth was Salle's crystal-violet lactose broth. Since fewer organisms were necessary to give a positive presumptive test with *Bact. coli* and *Bact. aerogenes* when lactose broth was employed than when the other presumptive test media were used, it would appear that these media are able to inhibit *Bact. coli* and *Bact. aerogenes* to a considerable degree. It is also interesting to note that in every case fewer coli than aerogenes were required to give a positive presumptive test.

TABLE 1

Number of organisms from emulsions required to give a positive presumptive test*

(Average of four series of examinations)

LACTOSE BROTH		SALLE'S MEDIUM		BRILLIANT-GREEN LACTOSE PEPTONE BILE		DOMINICK AND LAUTER MEDIUM	
Coli	Aerogenes	Coli	Aerogenes	Coli	Aerogenes	Coli	Aerogenes
2	10	>950	>1400	10	21	100	>1400

* The *Bact. coli* emulsions were made from twenty-four-hour agar slants of organisms freshly isolated from human feces.

The *Bact. aerogenes* emulsions were prepared from a laboratory strain of the organism.

COLLECTION AND ANALYSIS OF WATERS

In all, 50 samples of water were collected during the winter and spring of 1932–1933. The samples were collected aseptically in sterile, glass-stoppered bottles of 1 liter capacity, and transported immediately to the laboratory for examination. Nearly all of the samples were derived from sources in Metropolitan Boston. Two samples were obtained in New Hampshire, but these were thoroughly refrigerated in transit and were examined within twelve hours after collection. The waters are classified from the standpoint of source and sanitary quality in table 2. The sanitary quality was determined from a sanitary survey of the surroundings and a knowledge of the potential and actual pollution the water in question experiences from the source to the point of sampling.

From table 2 it is apparent that at least 50 per cent of the waters used for analysis were of doubtful or definitely unsatisfactory sanitary quality. Only 16 per cent of the waters were treated or finished supplies, the remainder being untreated except for the purification effected by storage and filtration through the soil to form a ground water supply. The majority of the waters were natural supplies derived from surface sources.

TABLE 2

Classification of waters examined according to source and sanitary quality

SOURCE	NUMBER	POLLUTED	DOUBTFUL	SAFE
Brooks	4	4	0	0
Lakes	6	3	1	2
Ponds	10	8	1	1
Rivers	8	7	1	0
Wells	7	0	0	7
Treated waters	8	0	0	8
Stored waters	7	1	0	6
Totals	50	23	3	24

After the waters were brought to the laboratory they were inoculated into the four presumptive test media used for comparison in the amounts decribed under "procedure." Each positive and doubtful presumptive test was studied on eosin methylene-blue agar for the partially confirmed test, and the positive partially confirmed tests were studied in lactose broth for the completed test. An agar slant was also prepared from each partially confirmed test and studied microscopically for the presence of Gram-negative, non-spore-forming, rod-shaped organisms resembling *Bact. coli.* A completed test coupled with a positive microscopic examination was considered confirmatory evidence of the presence of *Bact. coli.* (See table 3.)

TABLE 3

Summary of positive presumptive tests obtained on 50 water supplies classified according to type

TYPE OF WATER	NUMBER OF INOCULATIONS IN EACH MEDIUM	NUMBER OF POSITIVE PRESUMPTIVE TESTS REGARDLESS OF DILUTION				NUMBER OF COMPLETED TESTS			
		Lactose broth	Salle's medium	Brilliant-green lactose peptone bile	Dominick-Lauter medium	Lactose broth	Salle's medium	Brilliant-green lactose peptone bile	Dominick-Lauter medium
Brooks	120	101	88	101	98	79	64	90	96
Lakes	180	28	22	34	23	12	14	19	16
Ponds	300	95	61	96	69	70	33	77	45
Rivers	240	150	113	132	124	101	94	122	109
Wells	210	0	0	3	0	0	0	3	0
Treated waters	240	0	0	0	0	0	0	0	0
Stored waters	210	1	3	2	4	1	2	1	4
Total	1,500	375	287	368	318	263	207	312	270
Per cent of all samples, giving positive presumptive tests and per cent of positive presumptive tests showing positive completed tests		25.0	19.1	24.5	21.2	70.2	72.2	84.9	85.0

It will be seen that with the wide variety of waters under consideration lactose broth was the most sensitive presumptive test medium employed. It should be noted, however, that brilliant-green lactose peptone bile gave results almost as satisfactory. A similar condition obtained when suspensions of fecal coli in water were examined. The results obtained with Dominick-Lauter medium and with Salle's crystal-violet lactose broth were less satisfactory. There is no reason to believe that the smaller proportion of positive presumptive tests with these two media was due to the elimination or suppression of false-positive presumptive tests obtained with lactose broth and brilliant-green lactose peptone bile, especially as the proportion of completed tests made from brilliant-green lactose peptone bile was as high as the proportion obtained from the Dominick and Lauter medium. It is also noteworthy that the proportion of completed tests made from positive presumptive tests in Salle's medium was practically equivalent to the proportion obtained from lactose broth.

Although the proportion of completed tests made from positive presumptive tests in lactose broth was the smallest obtained, it does not follow that the higher proportion of positive presumptive tests obtained with this medium was due to organisms other than *Bact. coli*. It must be remembered that the completed tests were not made until twenty-four to forty-eight hours after the analysis of the water was initiated and that the members of the *Bact. coli* group may have been inhibited or overgrown during this period. Thus, it might be possible to obtain a positive presumptive test in lactose broth due to *Bact. coli* without obtaining a positive completed test. By shortening the period of incubation and by buffering the lactose broth medium adequately, it ought to be possible to throw some light on this aspect of the water examination for *Bact. coli*. Work to that end has already been initiated.

Dividing the data in table 3 according to the results obtained for finished and unfinished waters does not alter the results. Assuming that the brook, lake, pond and river waters were unfinished waters, there were 840 inoculations into each presumptive test medium. Of this number lactose broth gave a positive presumptive test in 374 cases (44 per cent), Salle's medium, 284 positives (33 per cent), brilliant-green lactose peptone bile 363 positives (43 per cent), and Dominick-Lauter medium 304 positives or 36 per cent. Of the positive presumptive tests in lactose broth, 70 per cent were completed; in Salle's medium 72 per cent were completed; in brilliant-green lactose peptone bile, 85 per cent were completed; and in Dominick-Lauter medium, 87 per cent were completed. Among the finished waters which included the treated supplies, the stored waters and the well waters, there were 760 inoculations in each presumptive test medium, but the number of positive presumptive tests was so small that statistically reliable conclusions could not be drawn. In general, however, lactose broth was less satisfactory as a presumptive test medium than any of the others employed.

From table 4, the presumptive test media that give the highest proportion of doubtful tests are Salle's crystal-violet lactose broth and standard lactose broth. There is, however, a noteworthy difference in the proportion of completed tests obtained from the doubtful presumptive tests. The Dominick and Lauter medium which gave the smallest proportion of doubtful presumptive tests showed the highest proportion of completed tests, the total being 53 per cent. Salle's medium which gave the highest proportion of doubtful tests also showed a very high proportion of completed

TABLE 4

Summary of doubtful presumptive tests obtained on 60 water supplies classified according to type

TYPE OF WATER	NUMBER OF INOCULATIONS IN EACH MEDIUM	NUMBER OF POSITIVE PRESUMPTIVE TESTS REGARDLESS OF DILUTION				NUMBER OF COMPLETED TESTS			
		Lactose broth	Salle's medium	Brilliant-green lactose peptone bile	Dominick-Lauter medium	Lactose broth	Salle's medium	Brilliant-green lactose peptone bile	Dominick-Lauter medium
Brooks	120	11	11	10	5	5	11	2	4
Lakes	180	19	28	25	26	7	14	8	15
Ponds	300	55	58	39	47	17	29	13	23
Rivers	240	45	50	53	37	12	18	25	26
Wells	210	0	0	0	1	0	0	0	0
Treated waters	240	13	0	1	0	0	0	0	0
Stored waters	210	16	17	14	14	2	3	0	1
Total	1,500	159	164	142	130	43	75	48	69
Per cent of all samples, giving positive presumptive tests and per cent of positive presumptive tests showing positive completed tests		10 6	10 9	9.5	8 7	27 0	45 7	33 8	53.0

tests (45.7 per cent). Brilliant-green lactose peptone bile was third in the proportion of completed tests obtained from the doubtful presumptive tests, and lactose broth showed the smallest proportion in this comparison.

It would seem from the data presented in table 4 that the organisms producing a doubtful presumptive test in lactose broth are either non-coli like in identity and therefore yield spurious presumptive tests or that they are members of the colon group and are inhibited or destroyed during the long period of incubation. There is nothing in this investigation which can throw any light on this question. On the other hand, it seems logical to say that the presumptive test media containing the inhibitory dyes used in this investigation are able to weaken or destroy the coli-like organisms present in water, for a positive or doubtful presumptive test yields a greater proportion of completed tests than the per cent obtained with standard lactose broth.

The conclusions that can be drawn from table 5 are essentially similar to those recorded in the discussion of tables 3 and 4. Lactose broth is the most delicate presumptive test medium employed, with brilliant-green lactose peptone bile being a close second. Taking the positive and doubtful presumptive tests into consideration simultaneously, it must be concluded that more completed tests are obtained from the Dominick-Lauter medium than from any other presumptive test medium employed. This must not be interpreted to mean, however, that the Dominick-Lauter medium is superior to the others as a presumptive test medium. It may really be inferior since it may be eliminating certain organisms in the sanitary water analysis that are of sanitary significance. While lactose broth as a presumptive test medium gave a positive or doubtful test in 86 instances in excess of the Dominick-Lauter medium, it yielded 33 fewer completed tests. On the other hand, brilliant-green lactose peptone bile gave only 24 fewer combined tests than lactose broth, but yielded 54 more completed tests and gave a total number of completed tests in excess of that obtained with any other medium.

Since the object of the sanitary water analysis is to detect pollution, and since in the presence of *Bact. coli* of recent intestinal origin, lactose broth proved to be more sensitive as a presumptive test medium than the other media used for comparison, it would seem undesirable to abandon the use of lactose broth for this purpose at the present time. This is especially true since it may be possible by shortening the period of incubation and adequately buffering the lactose broth, to increase materially the proportion of completed tests obtained from the positive and doubtful presumptive tests. The excellent results obtained with brilliant-green lactose peptone bile in this investigation, both as a sensitive presumptive test medium and as a medium yielding a high proportion of completed tests indicate the desirability of further work with this medium and the possibility of using it eventually as the standard presumptive test medium.

TABLE 5

Summary of positive and doubtful presumptive tests combined obtained on 50 water supplies classified according to type

TYPE OF WATER	NUMBER OF INOCULATIONS IN EACH MEDIUM	NUMBER OF POSITIVE PRESUMPTIVE TESTS REGARDLESS OF DILUTION				NUMBER OF COMPLETED TESTS			
		Lactose broth	Salle's medium	Brilliant-green lactose peptone bile	Dominick-Lauter medium	Lactose broth	Salle's medium	Brilliant-green lactose peptone bile	Dominick-Lauter medium
Brooks	120	112	99	111	103	84	75	92	100
Lakes	180	47	50	59	49	19	28	27	31
Ponds	300	150	119	135	116	87	62	90	68
Rivers	240	195	163	185	161	113	112	147	135
Wells	210	0	0	3	1	0	0	3	0
Treated waters	240	13	0	1	0	0	0	0	0
Stored waters	210	17	20	16	18	3	5	1	5
Total	1,500	534	451	510	448	306	282	360	339
Per cent of all samples, giving positive presumptive tests and per cent of positive presumptive tests showing positive completed tests		35.5	30 0	34 0	29.8	57 4	62.5	70 6	75 7

SUMMARY

Fifty waters, mostly unfinished and of surface origin, were studied in lactose broth, brilliant-green lactose peptone bile, Salle's crystal-violet lactose broth, and Dominick and Lauter's medium to compare the effectiveness of these media for presumptive test purposes. Freshly isolated strains of fecal *Bact. coli* and of laboratory cultures of *Bact. aerogenes* were prepared as bacterial suspensions and also used in this comparative study.

CONCLUSIONS

1. Lactose broth was the most sensitive presumptive test medium when freshly isolated strains of fecal *Bact. coli* were used. Brilliant-green lactose peptone bile was second, the Dominick and Lauter medium, third, and Salle's crystal-violet lactose broth, fourth.
2. The same arrangement held essentially when suspensions of *Bact. aerogenes* in water were employed.
3. When the waters were examined, lactose broth again proved to be the most sensitive presumptive test medium, with brilliant-green lactose peptone bile showing almost the same degree of sensitivity. The other media were less satisfactory in this respect.
4. The Dominick and Lauter medium yielded the greatest proportion of completed tests, the brilliant-green lactose peptone bile being almost as good. Numerically, brilliant-green lactose peptone bile yielded more completed tests than any other medium.
5. The superior results obtained with lactose broth as a presumptive test medium cannot necessarily be explained on the ground that it is more susceptible to spurious presumptive tests. The inferior results obtained with the other media may be due to the inhibition or destruction of the so-called weaker strains of *Bact. coli*, which may have sanitary significance.
6. In view of this possibility, lactose broth still seems to be the best presumptive test medium available. Efforts to improve its effectiveness by suitable buffering, by shortening the period of incubation and by other means should be made before it is abandoned.

7. The excellent results obtained with brilliant-green lactose peptone bile as a presumptive test medium and as a medium yielding completed tests indicate its great value in the sanitary water analysis. Further work is necessary, however, before it can be definitely accepted as a substitute for lactose broth as a presumptive test medium.

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BACTERIA DECOMPOSING ALGINIC ACID[1]

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Alginic acid is a complex carbohydrate belonging to the group of polyuronides; these yield, on hydrolysis with mineral acids, sugar acids, known as uronic acids, or mixtures of these acids with hexose or pentose sugars; purified alginic acid yields on hydrolysis only mannuronic acid. Alginic acid occurs abundantly in various marine algae, to the extent of 20 to 30 per cent of the total algal material. Although algal residues serve as food for a great variety of animals living both in water and on land, a large part of the residues are decomposed in nature by the action of microörganisms. The processes of their decomposition, especially the fate of the alginic acid complex, still remain to be determined.

In the course of a study of the decomposition of marine algal material, a number of bacteria capable of decomposing alginic acid were isolated from sea water, from phytoplankton and from the sea bottom. The decomposition of this compound was also studied in the case of land soils and several bacteria isolated; these differed, however, in certain of their physiological characteristics from the marine bacteria. It is important to add here that bacteria are the all-important agents concerned in the decomposition of alginic acid in nature. Even in soil, which is such a favorable medium for the growth of numerous fungi and actinomyces, the decomposition was carried out almost entirely by bacteria. The most active cellulose- and hemicellulose-decomposing fungi present in the soil were found to attack the alginic acid only to a limited extent. This was further substantiated by the fact that while active specific enzyme preparations were obtained from the bacteria, fungus enzymes, such as taka-diastase and pectolytic enzymes, had no effect upon the alginic acid.

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The following methods were used in the isolation of the bacteria from their respective habitats. One per cent solution of purified alginic acid (Schmidt and Vocke, 1926) prepared from different species of Fucus, largely *F. vesiculosus*, was dissolved in NaOH or KOH solution and the reaction adjusted to pH 7.0 to 7.2. Sodium nitrate and salts, in concentrations used in Czapek's solution were added. For the isolation of the marine bacteria, sea water or 3.5 per cent NaCl solution was used; for soil bacteria, distilled water.

The liquid media were inoculated with sea water, a piece of algal growth, phytoplankton, marine mud or sand, or with land soil. After bacterial development took place, as shown by the formation of turbidity in the medium, by pellicle or gas formation, the crude cultures were plated out on the corresponding agar media, and the bacteria isolated. A number of cultures were obtained; these were tested for their ability to decompose alginic acid, and only the most active forms selected for further study.

The determination of the decomposition of alginic acid by bacteria presents no difficulty in the case of distilled water or salt water media; the residual alginic acid is precipitated by means of a mineral acid or by addition of a solution of a calcium salt. In the case of the sea water medium, a large part of the alginic acid is precipitated in the medium by the calcium and magnesium of the water; as a result of the development of specific bacteria, the precipitate gradually disappears, due both to the action of the organisms and to that of the enzyme, alginase, which they produce abundantly.

The various alginic-acid-decomposing bacteria so far isolated both from the sea and from soil were found to be Gram-negative, non-spore-forming rods; they vary, however, considerably in their physiological properties. Some are more specific than others: some are able to attack, in addition to alginic acid, starch and other carbohydrates and even agar-agar, while others are more strictly limited to alginic acid as the chief source of energy. The soil bacterium described here produces gas actively from alginic acid, while the marine bacteria do not. Some of the bacteria produce pigments, while others do not. They also vary in the rapidity of alginic acid decomposition. All the bacteria stain better with aqueous fuchsin or gentian violet than with carbol erythrosin; with the latter stain, the cells appeared much smaller and more coccoid.

A description of four types of bacteria selected as representing the alginic-acid-decomposing organisms in nature is reported here. The forms most closely related to this group are the agar-liquefying bacteria. The *B. gelaticus* of Gran and especially the form later described by Lundestad (1928), under the name of *B. granii*, seem to be related to the first organism, namely *Bact. alginovorum*. However, the growth characteristics of the two organisms are sufficiently different and the activity of the latter in decomposing alginic acid is so marked that a detailed description of this form is justified. The *Bact. fucicola*, with its specific characteristics and pigment production, has evidently not been described before. *Bact. alginicum* may have been referred to in the various descriptions of marine, especially agar-liquefying, bacteria, such as those of Angst (1929), but because of the fact that these descriptions are so imperfect, it was felt best to describe this form as well.

BACTERIUM ALGINOVORUM N. SP.

Rods with rounded to almost elliptical ends, especially when single. Occurring frequently in twos and even in chains, 1.5 to 2.0 by 0.75 to 1.2 microns in size. Non-spore-forming. Actively motile. Gram-negative. Capsule-forming. Stains well with aqueous fuchsin. Appears as a short coccoid form in carbol erythrosin stain.

Alginic acid plate: colony large, white in appearance with coarse granular center, entire margin. Clears up turbidity caused by the alginic acid on plate. No odor.

Alginic acid liquid medium: heavy pellicle formation. Active production of an enzyme, alginase, which brings about the disappearance of the alginic acid precipitate in sea water medium. On salt water medium, a slimy pellicle of a highly tenacious nature is produced, the whole medium later turning to a soft jelly.

Sea water gelatin: active and rapid liquefaction in two to six days, at 18°C.; highly turbid throughout the liquefied zone.

Sea water glucose broth: abundant uniform turbidity, with surface pellicle; some strains give heavier turbidity and others heavier pellicle.

Litmus milk containing 3.5 per cent salt: no apparent growth.

Potato moistened with sea water: moist, spreading growth, ivory-colored; heavy sediment in free liquid at bottom.

Starch plate: abundant, cream-colored slimy growth; extensive diastase production.

Agar liquefaction: extensive softening of agar, no free liquid.

Aerobic, although able to grow under reduced oxygen tension.

Optimum temperature: 20°C.

Habitat: isolated from sea water, sea bottom sediments and from surface of algal growth in the sea. Very common in the sea.

BACTERIUM ALGINICUM N. SP.

Rods short to almost spherical, 0.6 to 1.0 micron in diameter. Non-spore-forming. Sluggishly motile. Gram-negative. Capsule-forming. When stained with aqueous fuchsin, the organism is found to be definitely a rod with rounded ends, occurring in twos. In carbol erythrosin stain, it is distinctly coccoid.

Alginic acid plate: white, finely granulated colonies, with entire margin. Does not clear up the turbidity in plate, due to limited production of the enzyme alginase. Odor formed, resembling that of old potatoes.

Alginic acid liquid medium: thin pellicle, weak alginase formation. Grows less rapidly and decomposes alginic acid less actively than previous organism.

Sea water gelatin: thin growth throughout gelatin stab, no liquefaction in seven days at 18°C.

Sea water glucose broth: uniform but very limited turbidity; no pellicle; no sediment.

Litmus milk containing salt: no apparent growth.

Potato moistened with sea water: moist, spreading growth, cream-colored; heavy sediment in free liquid at bottom.

Starch plate: limited, pale blue growth; no diastase.

Agar liquefaction: none.

Aerobic.

Optimum temperature: 20°C.

Habitat: isolated from sea water, and from surface of algal growth. A common form.

BACTERIUM FUCICOLA N. SP.

Rods, short, with ends rounded to almost coccoid; slightly curved; occurs singly and in twos. Does not stain evenly, 1.0 to 1.5 by 0.6 to 1 micron in size. Gram-negative. Actively motile, with twirling motion.

Alginic acid plate: colonies finely granular, entire; at first whitish, turning in three to five days brown, and later almost black, producing a deep brown soluble pigment.

Alginic acid liquid medium: limited growth on surface in the form of a pellicle. Frequently produces no growth at all.

Sea water gelatin: active liquefaction; no growth in stab; thin, fluorescent growth throughout liquefied zone.

Sea water glucose broth: faint turbidity; no pellicle, no sediment.

Litmus milk containing salt: no apparent growth.

Potato moistened with sea water: no growth.

Starch plate: no growth.

Agar liquefaction: positive, although limited; only softening of agar.

Aerobic.

Optimum temperature: 20°C.

Habitat: isolated from sea water taken from George's Bank, near the surface of the sand bottom. Rare occurrence.

BACTERIUM TERRESTRALGINICUM N. SP.

Long rods, with somewhat rounded ends, occurring usually single, but also in twos, and occasionally in chains of shorter rods, 1.5 to 2.5 by 1.0 to 1.5 microns. Gram-negative. Motile. Granular.

Alginic acid plate: colonies small, whitish in appearance with a slight metallic sheen.

Alginic acid liquid: medium at first clouded. Later, a pellicle is formed on surface of medium, which is soon broken up due to active gas formation. Reaction of medium becomes slightly alkaline.

Gelatin medium: slow growth throughout stab; slow liquefaction at surface of medium, at 18°C.

Glucose broth: abundant turbidity; some sediment; no pellicle; slightly fluorescent.

Litmus milk: acid; milk coagulated; only limited digestion of coagulum.

Potato: abundant, pinkish-colored, compact, dry growth on surface of plug, the rest of plug becoming grey, with tendency to darkening.

Starch plate: limited growth along streak; no diastase.

Agar liquefaction: none.

Aerobic to facultative anaerobic.

Optimum temperature: 30°C.

Habitat: soil.

In order to illustrate the action of the different bacteria upon alginic acid and upon crude algin, certain data are reported in tables 1 to 3. The term algin is applied to the washed acid precipitate of the dilute ammonium hydroxide extract of the algal material. The crude algin preparation contained 51.8 per cent uronic acid, 2.1 per cent ash and 1.66 per cent nitrogen.

In the first experiment, 100-cc. portions of sea water medium containing 1 gram of the air-dry alginic acid preparation, in the form of a potassium salt, were placed in flasks. A stream of CO_2-free air was passed above the cultures. The CO_2 produced by the organisms was absorbed in standard $Ba(OH)_2$ solution and the excess of the latter titrated with standard oxalic acid. Decomposition of the alginic acid was allowed to proceed, at room temperature, for twenty-one days. The results (table 1) show that the purified alginic acid is decomposed by the various bacteria. However, the extent of decomposition of the alginic acid in the crude algin preparations was much less, with greater variation among the different bacteria (table 2). In the last experiment, 300-mgm. portions of the algin preparation in the form of the potassium salt were used in 100-cc. portions of medium.

TABLE 1

Decomposition of purified alginic acid by marine bacteria

Incubation 21 days

BACTERIAL CULTURE	CO_2 GIVEN OFF	RESIDUAL ASH-FREE ALGINIC ACID	ALGINIC ACID DECOMPOSED
	mgm. of C	*mgm.*	*mgm.*
Control	0.15	935	
Bact. alginicum	113.93	308	627
Bact. alginovorum, No. 4	98.63	160	775
Bact. alginovorum, No. 10	101.56	150	785
Bact. fucicola	122.13	175	760

TABLE 2

Decomposition of crude algin by marine bacteria

Incubation 21 days

BACTERIAL CULTURE	CO_2 GIVEN OFF	RESIDUAL ASH-FREE ALGIN	ALGIN DECOMPOSED
	mgm. of C	*mgm.*	*mgm.*
Control	0.40	266	
Bact. alginicum	43.21	181	85
Bact. alginovorum, No. 4	56.86	118	148
Bact. alginovorum, No. 10	44.92	146	120
Bact. fucicola	46.26	156	110

TABLE 3

Decomposition of alginic acid by Bact. terrestralginicum

	URONIC ACID ANHYDRIDE		REACTION OF CULTURE
	Left	Decomposed	
	mgm.	*mgm.*	*pH*
Control	443.5		6.5
Bacterium	343.3	100.2	7.2

The rapidity of decomposition of the alginic acid by the soil organism was then tested. Fifty-cubic centimeters of distilled water medium, each containing 0.5 gram of the air-dry alginic acid preparation, in the form of a sodium salt, were inoculated with the bacterium and incubated at 28°C. for twenty days. No attempt was made to measure the CO_2 given off in the growth of this organism. The amount of uronic acid anhydride decomposed by the culture was determined. However, the decrease in total uronic acid content can not be taken as a true index of decomposition of the original alginic acid, since considerable hydrolysis of complex to smaller mannuronic acid units took place, without complete decomposition. The growth of the soil organism was accompanied by active gas evolution.

The various bacteria, both of marine and soil origin, were also found capable of decomposing alginic acid in the original Fucus material.

SUMMARY

The decomposition of alginic acid, a polyuronide occurring abundantly in marine algae, was found to be brought about largely by the action of certain specific bacteria, and only to a very limited extent by other microörganisms, such as fungi. Specific bacteria capable of decomposing alginic acid occur abundantly in sea water, in marine plankton, in the sea bottom and in land soils.

Four species of bacteria are described in this paper, three of which were isolated from the sea and one from the soil.

These bacteria decompose alginic acid actively, not only in purified preparations, but also in the original algal material.

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STUDIES ON BACTERIOPHAGE

I. RELATION OF BACTERIOPHAGE AND OTHER LYTIC AGENTS TO NEGATIVE STOOL CULTURES. STUDY OF TWO CASES[1]

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Negative cultures from stools of patients convalescing from bacillary dysentery were first obtained by d'Herelle in 1916, and this phenomenon led to the discovery of the dysentery bacteriophage. Negative cultures from stools of patients convalescing from typhoid fever due to the presence of potent anti-coli bacteriophages are not discovered on record. An attempt to explain such negative stool cultures by the presence of an anti-coli bacteriophage as well as by other lytic substances present in the intestinal tract during convalescence is the subject of this communication.

Two cases of clinical typhoid fever, the subject of this report, were admitted to the Beth-El Hospital, Brooklyn, during the year 1932–1933. The cases were as follows:

CASE I

B., forty-eight-year-old white female was admitted to the hospital with headache, abdominal pains and a macular abdominal rash, rose spot in type, a mounting temperature and chills (at onset) of twelve days duration. Spleen was not palpable, the remainder of examination was negative. Widal showed strong agglutination with *B. typhosus* in dilution 1:100, slight at 1:200 on admission; more marked at 1:200 three days later; no agglutination with paratyphoid A and B or *B. proteus* X_{19} was present. The disease was deemed too far advanced for blood culture investigation in the light of the clinical picture and a positive Widal. There was no history of antecedent typhoid fever or anti-typhoid inoculation. The patient made an uneventful recovery and was discharged after a month.

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[1] This and the immediately succeeding paper are based on a thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in New York University.

Stools were sent to the laboratory regularly for culture and isolation of typhoid organism. The medium employed was brilliant-green agar (Krumwiede). The first stool after admission was sent to the laboratory on April 27 after about two weeks of illness. After twenty-four hours *B. coli* and *B. pyocyaneus* "E(a)"[2] colonies developed on agar plates. Stools cultured on April 29 and on May 1 gave the same results: *B. coli* and *B. pyocyaneus* "E(b)" and "E(c)" respectively. Typhoid organisms could not be detected in any of these stools. The next stool, cultured on May 3, gave an entirely unexpected result. After twenty-four, forty-eight, seventy-two hours and thereafter, all four brilliant-green agar plates remained sterile indefinitely, although the method of culturing of this stool was identical with the preceding ones and the medium used belonged to the same stock. The possibility of any technical mistake seemed to have been excluded.

An attempt was made to establish the presence of a bacteriophage in the stool of the patient as a possible cause for the negative culture. If present, the bacteriophage probably reached its maximum virulence for the organism or organisms in question on May 3 and was most likely present in the stool on May 1, prior to the negative culture and in stools following it.

From the stool culture plates of May 1, a filtrate "B" was prepared. *B. coli* "B_1",[3] *B. pyocyaneus* "E(a)", "E(c)" gave negative results after three successive passages with filtrate "B", but *B. pyocyaneus* "E(b)" which differed from the two other strains by the brighter blue-green color exhibited, after twenty-four hours, and more pronouncedly after forty-eight hours incubation with the same filtrate, more or less circular pocketed areas, on the lower part of the agar slant. By transmitted light these pockets appeared as transparent areas against the blue-green culture. By reflected light, they presented an iridescent effect, showing bronze, yellow, green and blue glints and silvery flecks. In bouillon, a partial clearing took place, the clearing never becoming complete. The filtrate-free controls on agar and in bouillon showed the same phenomenon. At first, the latter was considered the result of contamination of culture with the lytic principle; the incompleteness of the lysis in the test tubes, on the other hand, was interpreted as due to the slight virulence of the lytic principle. It followed then, that if the lytic principle was one of d'Herelle's (a true bacteriophage), its power should increase by successive passages at the expense of its homologous culture, and the supposedly contaminated culture could be purified by plating and successive transfers. For this purpose, subcultures were made from areas of agar slant culture apparently unaffected (by the lytic principle) in bouillon and plated. Almost all of the colonies on the plate seemed to be affected. At the same time no increase in the power of the lytic principle could be demonstrated after ten successive passages at the expense of the same strain. The amount of filtrate added influenced neither the number of iridescent spots on agar, nor the rapidity of the lytic process in bouillon tubes.

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[2] "E(a)", "E(b)", "E(c)" are the strains of *B. pyocyaneus* isolated from three successive stools.

[3] "B_1" is a strain of *B. coli* isolated from culture of May 1.

From these observations and from those of d'Herelle (1926), Hadley (1924), Okuda (1923), Lisch (1924), Pesch and Sonnenschein (1925) it seemed that this lytic agent of pyocyaneus cultures was more clearly analogous to the Twort phenomenon than to the lytic principle of d'Herelle.

But whatever this lytic agent of *B. pyocyaneus* might be, it exercised a destructive effect on the pyocyaneus organism "E(b)". In addition, *B. pyocyaneus* which was invariably isolated from all stools of "B" previous to the negative one, never appeared in any of the stool cultures that followed it.

A more difficult problem was presented by the absence of living *B. coli* from the stool on May 3. It could not be proved that the lytic agent of *B. pyocyaneus* "E(b)" might have been responsible also for the lysis or, at least, attenuation of the *B. coli* organism since the filtrate was apparently free from that substance. Hadley (1924), however, has found that this lytic agent of pyocyaneus cultures is active also against *B. coli* and *B. typhosus*, among other organisms.

Another possibility was that an anti-coli bacteriophage did exist in the stool filtrate, but that the failure to discover it was due to the resistance of the isolated strain of *B. coli* "B_1" to that bacteriophage.

According to d'Herelle, bacteriophages virulent for *B. coli* normally exist in the intestines of all animals. They are not always discovered because *B. coli* exist there in the form of resistant, non-resistant, and mixed strains. Therefore in the second attempt to isolate an anti-coli bacteriophage many strains of *B. coli* from successive stools were tested with the filtrates.

On May 5, 8, and 12, stools were cultured and filtrates prepared. After twenty-four hours incubation on all plates made thereafter a normal growth of *B. coli* appeared. No *B. pyocyaneus* or *B. typhosus* colonies were present. All the filtrates including the first one that was inactive with *B. coli* "B_1", were tested against 10 strains of *B. coli* isolated and purified from successive stools. The results revealed the presence of a potent anti-coli bacteriophage to which all strains were not equally susceptible. Strains "B_2", "B_3", "B_5", "B_7", "B_9" were acted upon by the bacteriophage in various degrees; "B_1", "B_4", "B_8", "B_{10}" were resistant to its activity. It is clear that if only the last strains had been brought in contact with all the filtrates the results would be misleading. The failure to obtain an anti-coli bacteriophage with the first filtrate was apparently due to the resistance of the single strain "B_1" employed and not to the absence of a bacteriophage.

The isolated substances from stools specimen "B" described above, namely, the lytic agent for *B. pyocyaneus* "E(b)" and the anti-coli bacteriophage, did not give complete and permanent dissolution of the organisms in question when brought into contact with their filtrates, thus hindering a possible explanation of the negative culture of May 3. The anti-coli bacteriophage after 6 hours of incubation produced complete clearing on agar slant with some of the strains (“B_2”) and almost complete dissolution in bouillon, but gave secondary cultures when incubated over night.

For the absence of *B. coli* in the negative culture only one explanation is possible. It appears that various isolated colonies of *B. coli* differed in resistance to the activity of the phage. It will be shown later that, due to the method of colony purification and consecutive serial passages of the organism in contact with bacteriophage, the resistance of the strain decreased. It can be assumed that either this particular strain of *B. coli* was a susceptible strain or that the coli bacteriophage reached its maximal virulence on May 3 so that the coli organisms were completely destroyed without developing secondary cultures. In the later cultures of May 5, 8, 12, 15, 18, resistant strains of *B. coli* developed and secondary cultures appeared. This supposition would gain support if, by consecutive serial passages of *B. coli* “B” with its corresponding bacteriophage, the strain could be rendered completely susceptible, or if another strain of *B. coli* could be found which would give complete and permanent dissolution in contact with the bacteriophage. The work was now continued in these two directions.

Bordet and Ciuca (1921), and Eliava and Pozarski (1921) give methods by which resistant strains are rendered non-resistant. Their methods are essentially the purification of the strain of organism from the admixed bacteriophage, followed by successive passages of the strain in the absence of the bacteriophage until the strain loses its acquired resistance to the bacteriophage. Another method of colony purification by Roux is highly recommended by d'Herelle (1926, p. 205).

Following these methods serial passages of the bacteriophage “B” were performed at the expense of the least resistant strain “B_2”. The resistance of the strain greatly decreased. After the seventy-fifth passage only 2 to 3 colonies appeared on an agar slant while the bouillon tube remained clear. These observations suggest that continued serial passages of *B. coli* “B_2” with bacteriophage “B” would eventually so attenuate the organism and enhance the phage virulence as to produce complete bacterial dissolution for indefinite periods.

Twelve heterologous strains of *B. coli* showed reactivity with the bacteriophage "B" on agar and some in bouillon, but this activity was low, so that the bacteriophage was quite specific for its homologous strain, which exhibited the greatest degree and the longest endurance of clearing.

The specificity of the anti-coli bacteriophage was also tested against the typhoid-dysentery group. All strains of *B. typhosus*, except one, showed reactivity with the bacteriophage. This may throw some light on the fact that the typhoid organism could never be isolated from the stools of "B". It may also indicate why the typhoid organisms could not be found in so many definite typhoid cases. All four strains of *B. dysenteriae* were acted upon by the anti-coli phage.

It was of special interest to see whether the *B. coli* bacteriophage "B" was active with *B. pyocyaneus* strains. A lytic agent of *B. pyocyaneus* was discovered in the culture "E(b)" which could only be partially responsible for the destruction of *B. pyocyaneus*, since this substance did not give complete lysis of the bacterial cells. If the anti-coli bacteriophage were also active with the *B. pyocyaneus* strains, especially with "E(b)",[4] an additional factor would be found to explain the disappearance of this strain in the negative cultures of May 3 and its absence in the following cultures. All strains of *B. pyocyaneus* were acted upon by the bacteriophage coli "B" and this activity was much higher than with the coli-typhoid group.

Finally, the titre of the bacteriophage "B" was determined by the method of d'Herelle (1926, p. 150). Both the dilution and plating methods were employed.

By the dilution method the activity of the bacteriophage "B" was 1×10^{-9}; by the plating method, 1×10^{-10}.

To summarize: two organisms were isolated from three consecutive stool cultures of patient "B": *B. coli* and *B. pyocyaneus*. From cultures antedating the negative one, both a lytic substance for *B. pyocyaneus* active against its homologous strain, as well as an anti-coli bacteriophage active against *B. coli* "B" were isolated.

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[4] Cultures "E(a)", "E(c)" by that time were destroyed by autolysis after 30 generations on agar.

It was found that the anti-coli bacteriophage exercised a twofold power: (1) for its own homologous strain, and (2) for *B. pyocyaneus.* The experiments by Hadley, (not repeated here), show that the lytic agent of pyocyaneus exercises an inhibitory power on the growth of *B. coli* and *B. typhosus.* It appears likely that an interaction of complicated factors of which only a few could be revealed in this work, effected the destruction of the organisms and produced the negative culture. *B. coli* is a normal inhabitant of the intestines, where, according to d'Herelle, it undergoes mutation under the influence of the bacteriophage. It is supposed that while the strain most susceptible to the anti-coli phage succumbed, the more resistant strains survived. *B. pyocyaneus,* on the other hand, not being a normal inhabitant of the intestines, was apparently destroyed and never again appeared in the stool cultures.

The absence of complete dissolution of the organisms in question *in vitro* required further explanation. In the intestinal tract, apparently, a complexity of factors existed. These factors were probably working at one time in a medium in which the products of growth were eliminated in the usual manner of animal metabolism, and therefore, did not exercise inhibitory power on the bacteriophage and other lytic agents. The bacteriophage was being produced continuously in unlimited amounts, not as is the case *in vitro.* If such were, approximately, the circumstances which created the negative culture on May 3, it was interesting to try to reproduce this original situation in an animal body.

It was originally planned to carry out the animal experiments in three ways:

Experiment 1. To introduce *B. coli* and the *B. coli* bacteriophage active against it intraperitoneally, peritoneal fluid from living animal and post mortem washings to be cultured on solid and liquid media, incubated and results observed after twenty-four to forty-eight hours.

Experiment 2. To introduce *B. pyocyaneus* with its lytic agent intraperitoneally; peritoneal fluid from living animal and *post mortem* washings to be cultured on solid and liquid media, incubated and results observed after twenty-four to forty-eight hours.

Experiment 3. To introduce *B. pyocyaneus* "E(b)" with its lytic substance, *B. coli* and the *B. coli* bacteriophage and *B. typhosus* and observe how the whole complex of factors will affect the peritoneal cultures after twenty-four to forty-eight hours.

The strain of *B. coli* "B_2" was passed through 3 guinea pigs to restore the original characteristics lost by *in vitro* passages and was used for animal experiments.

Experiment 1

For Experiment 1, three groups of guinea pigs were used.

The first group consisted of six healthy guinea pigs of 350 to 400 grams. Four animals (test) received 2 cc. of a twenty-four-hour suspension of *B. coli* "B_2" containing 500 million per cubic centimeter, plus 0.2 cc. of bacteriophage *B. coli* "B" intraperitoneally, two control animals receiving a similar amount of bacteria without bacteriophage. This amount and proportion of organisms and bacteriophage did not give dissolution of bacterial cells *in vitro*. Numerous Gram-negative bacilli were found in both groups, on smears and cultures from peritoneal punctures obtained every hour for six successive hours. There was no clearing in cultures from both groups. Cultures were also positive for *B. coli* from the peritoneal washings from all animals killed after twenty-four and forty-eight hours, but were absent from direct smears of all animals. All the guinea pigs were listless and showed a slight temperature fall on the day of inoculation.

With the second group of guinea pigs (same number of animals and of similar weight) the same procedure was followed except that the number of organisms of *B. coli* injected intraperitoneally was 150 million per cubic centimeter, an amount and proportion sufficient to give complete dissolution *in vitro* after four hours incubation, and secondary cultures after twenty-four hours. All cultures from peritoneal punctures were positive for *B. coli* after twenty-four hours, with partial clearing in those from the test animals and cloudiness in those from the controls. On agar slants cultures from test animals showed irregular "eaten out" colonies (Flattenformen of Gildemeister[6]) and regular circular colonies in cultures from control animals. Smears from peritoneal washings of two test and one control animal, killed after twenty-eight hours, were negative; cultures from heart blood were negative, but from the peritoneal cavity all were positive for *B. coli.* Two more test and one control animal killed after ninety-six hours gave exactly the same results. All animals were somewhat listless on the day of inoculation but their temperatures underwent no changes.

Five test animals in the third group received a suspension of *B. coli* "B_2" and bacteriophage *B. coli* "B" in the same amount and proportion as in the second group, but fresh amounts of bacteriophage (2 cc.) were added to 4 test animals twenty minutes after the first inoculation and successively thereafter for six hours, while to one test and two control animals the same amount of sterile physiological salt solution was administered similarly. No organisms appeared on smears from the hourly punctures of any animals. Peritoneal cultures after twenty-four hours were positive in all animals. Those obtained from test animals remained clear in bouillon for five to six days. Smears from these clear cultures showed ellipsoidal Gram-negative bacilli of varying dimensions. On agar, cultures from the test animals showed "Flattenformen" colonies; those from the test animal, receiving only one dose of bacteriophage gave partial dissolution in bouillon. Smears from test and control animals were negative. Peritoneal cultures obtained from test and control animals killed after twenty-eight and forty-eight hours were all positive for *B. coli.* Cultures from heart blood were negative. All animals were slightly sick and listless on day of inoculation only. Temperature fluctuations were not significant.

To summarize: The experiment on the first group of guinea pigs showed the same proportion of organisms and bacteriophage to be inactive *in vivo* as *in vitro.*

The experiments on the second group of guinea pigs showed unexpected results. Not only did cultures obtained from peritoneal punctures and post-mortem washings of test animals fail to give lasting dissolution of the bacterial cells, but the dissolution was never complete. They gave partial clearing in bouillon after four hours and remained unchanged after twenty-four hours. This indicates that some factor existed in the peritoneal cavity which exercised an inhibitory power on the bacteriophage. The experiments of Appelbaum and MacNeal (1932) showed that the activity of a bacteriophage is inhibited by purulent exudates. It is likely that the cause of incomplete dissolution in these cultures was due to this factor.

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[6] Change in form of colonies due to influence of bacteriophage.

The experiments on the third group of guinea pigs showed, that notwithstanding successive additions of bacteriophage, cultures obtained from peritoneal punctures and from *post-mortem* washings were all positive after twenty-four hours, although bouillon tubes remained clear for several days.

From these experiments, it is apparent that where a greater supply of phage was available for a longer period in the animal body (by repeated inoculation), there occurred a definite delay in the development of secondary cultures, and a distortion, if not total dissolution of organisms.

It is clear that experiment 1 failed to reveal the expected results. This may be due to the impossibility of artificially reproducing the exact environment which accompanies bacteriophagy in the intestinal tract of a convalescent patient. Even though these conditions could not be reproduced by simple technical methods, the findings suggested the validity of the original explanation for the negative culture.

Experiments 2 and 3 are now being carried on.

CASE II

K., eleven-year-old white female, was admitted to the hospital with a two-weeks history of mounting temperature, malaise, diarrhea and anorexia. Spleen was palpable on admission, a leukopenia and relative lymphocytosis was present; the remainder of the examination was negative. Widal examination was reported positive by New York Board of Health Laboratories three days before admission: was positive in the hospital in dilutions 1:100 and 1:200. There was no agglutination with paratyphoid A and B. and *B. proteus* X_{19}.

Nine stool specimens were cultured on brilliant-green agar and Endo media during a two-months period at varying intervals of time. The typhoid organism could not be detected in any of these stools. The results of the findings are given in table 1.

In the first stool specimen cultured on January 25, 1933, after twenty-four hours incubation an abundant growth of *B. coli* and *B. alkaligenes-fecalis* appeared. In a second stool specimen cultured on January 31, 1933, no growth appeared on any of the four seeded plates after thirty-six hours incubation; after forty-eight hours, a small number of *B. coli* colonies and a higher number of *B. alkaligenes-fecalis* developed on all agar plates. In order to demonstrate the presence of a bacteriophage in the intestinal tract *B. coli* colonies "K_1[6]", "K_2", "K_3", "K_4", "K_5" and a *B. alkaligenes-fecalis* colony purified by the usual method were tested with the filtrate prepared from a stool culture on February 1, 1933.

According to table 2 a potent anti-coli bacteriophage was present in the stool which gave complete dissolution in bouillon with strains "K_1", "K_3"; partial clearing with "K_4", "K_5", "K_6", "K_7" no activity with the other strains and partial clearing with *B. alkaligenes-fecalis*. Again, as in case I, the fact was confirmed that strains of *B. coli* exist in the intestines differing in susceptibility to the anti-coli bacteriophage.

The stool specimen cultures were examined for time of inhibition of growth of organisms, if any; the number of colonies appearing after prolonged incubation; the presence and virulence of bacteriophage in these stool filtrates, its duration in the intestinal tract; and the relationship between these factors, if any. Accordingly, filtrates, parallel to the cultures were prepared from all stool specimens thereafter. The results of these and subsequent stool-filtrate experiments are given in tables 1 and 2.

The isolated anti-coli bacteriophage "K" of the convalescent

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[6] "K_1", "K_2", "K_3", etc., are *B. coli* strains isolated from successive stools of patient K.

TABLE 1

Effect of successive stool filtrates "K" on strains "K" cultured in veal bouillon, pH 7.8

STRAINS, B. COLI "K"	STOOL FILTRATES OF THE FOLLOWING DATES																											
	February 1, 1933				February 5, 1933				February 9, 1933				February 14, 1933				March 10, 1933				March 19, 1933*				March 28, 1933			
	6 hours		12 hours		6 hours		12 hours		6 hours		12 hours		6 hours		12 hours		6 hours		12 hours		6 hours		12 hours		6 hours		12 hours	
	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
"K₁"	++++	0	++++	0	++++	0	++++	0	++++	0	++++	0	++++	0	++++	0	++	0	+	0	0	0	0	0	0	0	0	0
"K₂"	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
"K₃"	++++	0	++++	0	++++	0	++++	0	+++	0	+++	0	+++	0	+++	0	0	0	0	0	0	0	0	0	0	0	0	0
"K₄"	++	0	++	0	+	0	+	0	+	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
"K₅"	++	0	++	0	++	0	++	0	++	0	+	0	+	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0
"K₆"					+	0	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
"K₇"					+	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
"K₈"									0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
"K₉"													0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
"K₁₀"																	0	0	0	0	0	0	0	0	0	0	0	0
"K₁₁"																					0	0	0	0	0	0	0	0
"K₁₂"																					0	0	0	0	0	0	0	0
B. alkal fecalis†																									0	0	0	0

* Patient discharged from hospital after February 14, 1933; stools not obtainable until this date.

† *B. alkaligenis fecalis* grows slowly; after six hours no growth detected.

0 indicates negative results: no clearing in broth; no plaques on agar. + indicates lysis (25 per cent) in broth; few plaques on agar. ++ indicates lysis (50 per cent) in broth; many plaques on agar. +++ indicates lysis (75 per cent) in broth; agar slant almost clear. ++++ indicates lysis (100 per cent) in broth; agar slant perfectly clear.

TABLE 2

Showing findings of stool cultures of patient "K"

	NUMBER OF PLATE	NAME OF ORGANISM	STOOL SPECIMENS OF THE FOLLOWING DATES																	
			January 25, 1933		January 31, 1933		February 1, 1933		February 5, 1933		February 9, 1933		February 14, 1933		March 10, 1933		March 19, 1933		March 28, 1933	
			Hours of incubation																	
			24	48	36	48	36	48	36	48	24	48	24	48	24	48	24	48	24	48
Brilliant green agar	I	*B. coli*	*	*	0	3	0	5	0	7	8	10	60	75	†	†	*	*	*	*
		B. alkal. fecalis	†	†	0	20	0	1	0	0	0	0	0	0	0	0	0	0	0	0
		B. typhosus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	II	*B. coli*	75	100	0	1	0	3	0	3	5	4	12	23	30	42	69	76	69	80
		B. alkal. fecalis	30	40	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		B. typhosus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Endo media	I	*B. coli*	*	*	0	2	0	1	0	10	10	8	70	84	†	†	*	*	*	*
		B. alkal. fecalis	37	43	0	26	0	2	0	0	0	0	0	0	0	0	0	0	0	0
		B. typhosus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	II	*B. coli*	80	87	0	0	0	0	0	5	7	9	17	25	39	48	50	63	71	84
		B. alkal. fecalis	34	40	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		B. typhosus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* Innumerable colonies.

† Numerous colonies.

patient showed the following characteristics: there was a high activity with some of the isolated homologous *B. coli* strains, especially with strain "K_1", as shown by complete dissolution of the bacterial cells after four hours incubation at 32°C. The cleared tubes incubated over night, became cloudy because of the development of secondary cultures. This was apparently due to the fact that while the susceptible strains of *B. coli* were destroyed by the activity of the phage, the surviving strains were more resistant. Here, as in case I, an attempt was made to lower the resistance of *B. coli* "K" strains by colony purification. The results showed strain "K", to possess least resistance toward its homologous bacteriophage. At the expense of this strain, twenty-five serial passages of the bacteriophage K. were performed. That it was gaining in power with every passage, could be inferred from the decrease in time necessary to bring about complete dissolution of bacterial cells (from four hours to two hours) in bouillon, and the diminished number of colonies (secondary cultures) on agar slant after twenty-four hours incubation of organism and phage.

The virulence of bacteriophage "K" was determined by the method of d'Herelle (1926, p. 150).

By the dilution method it was 1×10^{-10}; by plating method, it was 1×10^{-11}.

SUMMARY AND CONCLUSIONS

Stool specimens of patient "K" did not reveal *B. typhosus* during two months of examinations. During convalescence, a potent anti-coli bacteriophage was isolated which was apparently responsible for the inhibition of growth of *B. coli* for one week, and for the disappearance of the *B. alkaligenes-fecalis* found in the stool. Whether the persistent absence of *B. typhosus* from all stool specimens was also due to the activity of this phage can only be suggested.

The bacteriophage "K" present in stool specimens retained its full power for six weeks, after which time its activity began to diminish. It disappeared after two months. During this period there was observed the gradual loss of susceptibility of all "K" strains to successive filtrates and the increasing number of *B. coli* colonies on agar plates after culturing of stools.

In comparing case I with case II from the bacteriological standpoint, points of resemblance as well as points of difference could be observed.

Both were cases of typhoid fever in which the typhoid organism could never be detected in any of the stool specimens cultured.

In both cases, stool specimen cultures before convalescence gave as abundant growth of the organisms isolated after twenty-four hours incubation. During convalescence, the stool cultures suddenly gave no growth. But, while in case I the negative culture remained so indefinitely, in case II the growth was inhibited for thirty-six hours, but a few colonies appeared on all plates after forty-hours.

In both cases, two types of organism were isolated from stools; in case I, *B. coli* and *B. pyocyaneus*, in case II, *B. coli* and *B. alkaligenes-fecalis*. In both cases, organisms other than *B. coli* were also acted upon by the anti-coli bacteriophage and they disappeared from the stool cultures as soon as the presence of the bacteriophage was manifested and never returned again.

Of the two cases, case II is more clear cut. In this case one can trace events and sequences in their logical developments. The normal culture of stool specimens before convalescence may be explained either by the absence of the bacteriophage or by its slight activity against the strains of intestinal bacteria. During convalescence, the bacteriophage acquired a high virulence. The susceptible strains of the intestinal flora succumbed to the activity of the bacteriophage, as shown by the negative culture. A few resistant strains of *B. coli* survived and a few colonies of *B. coli* appeared on agar plates after a much longer period of incubation than is usually necessary for the development of a normal strain of the same species. In time, due to symbiosis of *B. coli* with its bacteriophage in the intestinal tract, the former developed a resistance. Therefore, more colonies appeared on agar plates after stool culturing, but their number was still less than usual. The virulence of the bacteriophage was maintained until complete recovery. At this point it could no longer develop at the expense of its susceptible strains apparently because of their destruction. *B. coli* organisms then grew normally on culture media.

Despite discrepancies, case II confirms case I showing that a close relationship exists between the activity of the anti-coli bacteriophage in the intestinal tract of a convalescent patient, and the inhibition of growth of the intestinal bacteria. The phenomena of bacteriophagy in the two cases were essentially the same, the difference being of a quantitative rather than of a qualitative nature.

The author desires to express her thanks to Drs. William H. Park and Anna Williams of the New York City Department of Research Laboratories for helpful suggestion in the composition of these papers, and to Dr. Julius Klosterman of the Department of Bacteriology of New York University for suggestions throughout the work.

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STUDIES ON BACTERIOPHAGE

II. COMPARISON OF ACTION OF LYTIC AGENTS AND BACTERIOPHAGES UPON PYOCYANEUS CULTURES

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In the preceding paper, there was described a pyocyaneus culture "E(b)" isolated from the stool of a typhoid patient. It was characterized by a bright blue-green color, the spontaneous appearance of lytic pockets within the colonies on agar, and partial lysis in bouillon. The culture could never be separated from the lytic substance by Roux's method of colony purification (D'Herelle, 1926, p. 205); moreover, apparently unaffected colonies of the same culture, when transferred on agar slants or plates, yielded the same pocketed areas which showed the characteristic iridescent lustre in reflected light. Two other strains "E(a)" and "E(c)", isolated from the stool of the same patient at short intervals, were of a yellow-green color and did not show lysis on solid or in liquid media. Transfers made from these cultures gave homogeneous colonies of normal appearance on agar slants and plates and showed no lysis in bouillon.

B. pyocyaneus culture "D", isolated from urine, exhibited exactly the same characteristics as culture "E(b)".

B. pyocyaneus culture "J", isolated from another urine was of a yellow-green color at the time of isolation and did not show any signs of lysis. When transferred for ten generations on agar slants its chromogenic properties changed from yellow-green to bright blue-green and lytic pockets appeared with characteristic iridescent spots. Transfers from the lytic and non-lytic colonies invariably yielded lytic colonies.

Another culture of *B. pyocyaneus* "S", isolated from a spinal fluid, together with a meningococcus, showed two types of colonies on agar plates: bright blue-green colonies affected by the lytic substance as described above, and yellow-green homogeneous colonies. Hadley (1924) characterized the lytic strains of *B. pyocyaneus* cultures as possessing high growth energy and high enzymatic activity such as liquefaction of gelatin and casein, and pigment production. The high growth energy of the lytic colony "J" as compared with the resistant colony "C" is demonstrated in figures 2 and 3.

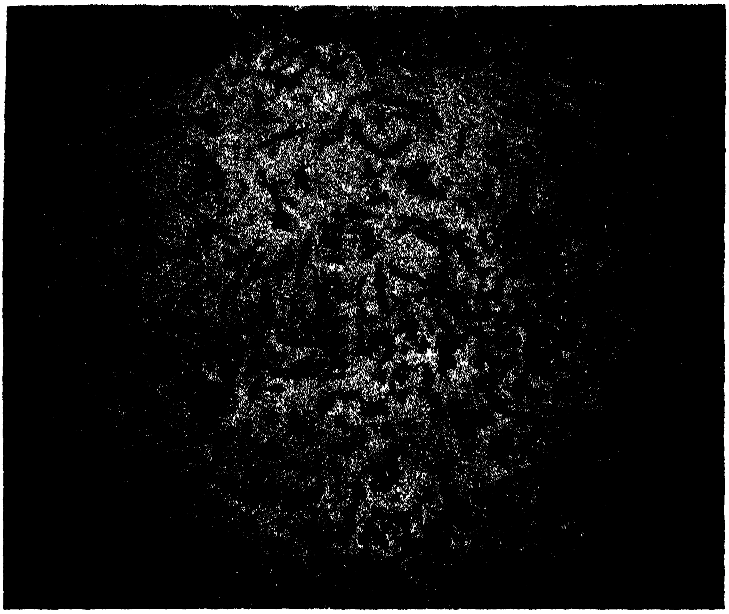

FIG 1. NEEDLE-LIKE CRYSTALS FROM A FORTY-EIGHT-HOUR LYTIC CULTURE OF B. PYOCYANEUS "D" SCATTERED OVER THE LYTIC AREAS AND SURROUNDING AGAR (× 20)

Another characteristic of the lytic strain is the deposition of crystals, mainly in cleared areas. Under low magnification they appear needle-like, lying singly (fig. 1), or grouped into stellate or radial aggregates (fig. 2). Crystals are not produced by the resistant strains.

Still another characteristic of the lytic agent of the pyocyaneus cultures consists in the fact that the lytic pockets, though transmissible in successive generations can be perpetuated only up to a

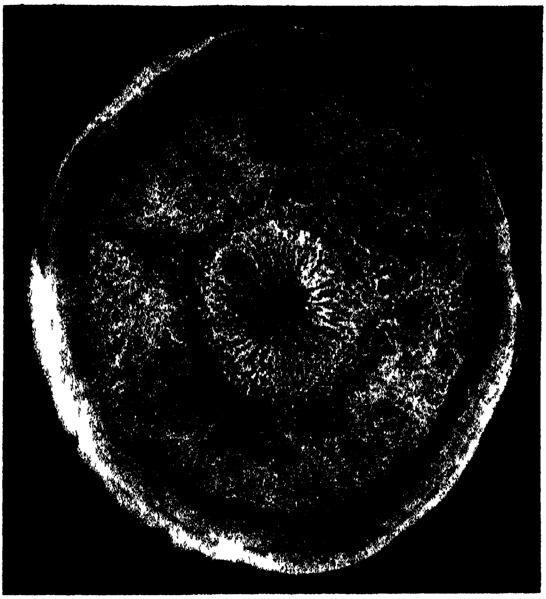

Fig. 2. A Colony of B. pyocyaneus "J" Seventy-two Hours Old

At the center of the colony star-like aggregates of crystals are deposited surrounded by lytic concentric areas. Note the size of the colony as compared with figure 3. (× 20.)

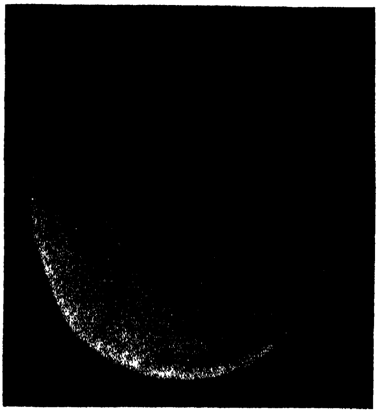

Fig. 3. Seventy-two-hour Normal Colony of B. pyocyaneus "C"

Note homogeneous construction and size of this colony as compared with figure 2. (× 20.)

certain limit, which in our experiments varied between thirty and forty generations. Out of 11 strains of *B. pyocyaneus* isolated at our laboratory from different sources, only four developed iridescent spots. Strain "E(b)" mentioned above, lost the lytic substance after 31 generations on agar slants. Two homologous strains "E(a)" and "E(c)", isolated from the stool of the same patient as "E(b)", but at short intervals, showed at the beginning no visible signs of lysis. However, "E(a)", after ten transfers, and "E(c)", after fifteen transfers, changed their chromogenic properties from yellow-green to bright blue-green and lytic pockets spontaneously appeared. This suggests that cultures "E(a)" and "E(c)" harbored the lytic agent in a latent state and that some factors (such as transfers on fresh media) may have stimulated its appearance. These two cultures after continued transfers on agar slants, lost their lytic properties after 33 generations.

A lytic strain of *B. pyocyaneus* "D" mentioned above, was destroyed during such transfers from generation to generation, the lytic agent being at the height of its activity.

In the *B. pyocyaneus* culture "J", the lytic substance was destroyed after 40 generations. When, however, after three weeks interval, the culture was transferred on fresh agar slant, the lytic pockets reappeared. Plated on agar, characteristic lytic colonies developed possessing a high growth energy (fig. 2). It is interesting to note that these lytic areas differed in their appearance from those of the same culture in its early manifestations, at which stage they possessed exactly the same appearance as that of cultures "E(b)", "D", "S".

In all the cases described above, the cultures, paralleling the loss of the lytic substance, underwent chromogenic changes; the brilliant green color and the lytic areas were maintained in their original integrity at the beginning, but after a certain period, the pigment began to diminish in amount and intensity; the lytic areas became less numerous until the pigment and the lytic areas disappeared from the cultures which again produced only a yellow-green pigment. Apparently there exists a close relationship between the lytic and chromogenic features of these pyocyaneus cultures. In the cultures "E(b)", "E(a)", "E(c)", "D", the lytic substance never returned in continued transfers; in "J", as stated above, the lytic agent reappeared, the color of the strain, however, remaining an unchanged yellow-green.

The lytic substance filtrates of *B. pyocyaneus* cultures, namely, "E(a)", "E(b)", "E(c)", "D", "J", "S" were tested against their homologous and 12 heterologous strains to determine the degree of specificity, if any, and to find a more susceptible strain at the expense of which complete lysis could be obtained. The results were recorded after ten successive passages. The filtrates in contact with the homologous and lytic heterologous strains, after twenty-four and more distinctly after forty-eight hours incubation, presented silvery flecks on agar slants and partial lysis in bouillon, but the controls which were filtrate-free gave exactly the same results. With the non-lytic heterologous strains the filtrates showed no activity whatsoever. Neither did the amount of filtrate added to the cultures influence the appearance or the number of the iridescent spots.

In comparing the lytic features of pyocyaneus cultures described above, with the lytic principle of d'Herelle, Hadley, although finding essential differences between the two phenomena, is inclined to consider them as belonging to the same order, varying rather in quantity and rate of action, than in quality. Canic also considered the silvery flecks on many pyocyaneus cultures as plaques of real pyocyaneus bacteriophages. Okuda (1923), however, differentiates two types of plaques in pyocyaneus cultures, "sharp holes" and "dark holes." He is likewise inclined to believe that there is no essential difference between the two phenomena. Lisch (1924) Pesch and Sonnenschein (1925) isolated real pyocyaneus bacteriophages and differentiated between the two phenomena.

From this brief review of the literature, it can be seen that, while some authors consider the phenomena displayed in pyocyaneus cultures as manifestations of the same principle, others regard them as entirely different.

An attempt to isolate a pyocyaneus bacteriophage from various sources was made. The sources were: pus of the ear (otitis media caused by *B. pyocyaneus*), urines, stools, a case of wound infection due to *B. pyocyaneus*, old agar and bouillon pyocyaneus cultures. Three successive passages were performed on all 11 strains of *B. pyocyaneus* isolated in our laboratories. They showed no appearance of plaques on agar, no dissolution in bouillon tubes, and no characteristic changes of colonies on agar plates. Filtrates prepared from old and young agar and bouillon cultures and tested with their homologous and heterologous strains gave the same negative results. All the strains isolated in our laboratories failed to reveal the presence of a bacteriophage.

A strain of *B. pyocyaneus* "I" of a light yellow-green color, however, obtained from Bellevue Medical School inoculated into bouillon over night and then transferred on an agar plate revealed a remarkable appearance after forty-eight hours incubation. The entire agar plate culture was distorted. The homogeneous growth as well as the isolated colonies were affected by some lytic principle. The clearing spots looked entirely different from the iridescent spots. They were smaller, had a more circular form, and did not show any iridescent appearance. Under the microscope no crystals were present. From this plate as well as from the original bouillon culture, filtrates were prepared and tested against their homologous strain "I", on agar slants and in bouillon tubes. After eight hours of incubation at 32°C. complete clearing appeared on the agar slant and in bouillon tube to which the filtrate was added, while clearing did not occur in the controls which were filtrate-free. In other words, the filtrates worked with homologous strains like any other bacteriophage.

The bacteriophage of pyocyaneus "I" isolated in this manner showed the characteristics of any other known bacteriophage. It passed through the Berkefeld filter, produced dissolution in bouillon and clearing on agar and non-iridescent plaques, in direct proportion to the quantities of filtrates admixed.

The virulence of the bacteriophage "I" was tested after the sixtieth passage. By titration method it was 1×10^{-8}. By plating method it was 1×10^{-9}.

Another *B. pyocyaneus* bacteriophage was isolated from a

TABLE 1*

Effect of addition of anti-pyocyaneus bacteriophage “I” to cultures of colon-typhoid-dysentery group†

B. TYPH. STRAINS	SOURCE	MEAT INFUSION AGAR pH 7.8								MEAT INFUSION BROTH pH 7.8							
		8 hours		12 hours		24 hours		48 hours		8 hours		12 hours		24 hours		48 hours	
		Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control	Test	Control
“S”	Stool	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
“C”		++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
“K”	Stool	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
“M”	Stool	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
Board of Health	Stool	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
“L”	Stool	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
“F”	Stool	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
“H”	Stool	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
Para A	Stool	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
Para B	Stool	+	0	+	0	+	0	+	0	+	0	+	0	+	0	+	0
B. dys. strains																	
Shiga	Board of Health	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
Sonne	Board of Health	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
Desert	Board of Health	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
Flexner	Board of Health	++	0	++	0	++	0	++	0	+	0	+	0	+	0	+	0
B. coli strains																	
“B” ‡	Stool	++	0	++	0	++	0	++		0	0	0	0	0	0	0	0
“T_1”	Urine	++	0	++	0	++	0	++	0	0	0	0	0	0	0	0	0
“D”	Urine	++	0	++	0	++	0	++	0	0	0	0	0	0	0	0	0
“W”	Urine	++	0	++	0	++	0	++	0	0	0	0	0	0	0	0	0
“K”	Urine	++	0	++	0	++	0	++	0	0	0	0	0	0	0	0	0
“S” ‡	Urine	++	0	++	0	++	0	++	0	0	0	0	0	0	0	0	0
“T_2”	Urine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
“Sh”	Urine	++	0	++	0	++	0	++	0	0	0	0	0	0	0	0	0
“Be”	Urine	+	0	+	0	+	0	+	0	0	0	0	0	0	0	0	0
“F_3”	Urine	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
“T_4” ‡	Stool	++	0	++	0	++	0	++	0	0	0	0	0	0	0	0	0
“K”	Stool	++	0	++	0	++	0	++	0	0	0	0	0	0	0	0	0
“K”	Stool									0	0	0	0	0	0	0	0

* Results are after four serial pooled passages.

† One-tenth cubic centimeter of phage added to test agar slant, 0.5 to 1.0 cc. of test bouillon.

‡ These bouillon cultures, however, when spread on agar plates revealed presence of few bacteriophage plaques.

0 indicates negative results, no clearing in broth, no plaques on agar. + indicates lysis (25 per cent) in broth, few plaques on agar. ++ indicates lysis (50 per cent) in broth, many plaques on agar. +++ indicates lysis (75 per cent) in broth, agar slant almost clear. ++++ indicates lysis (100 per cent) in broth, agar slant perfectly clear.

strain of *B. pyocyaneus* No. 284 obtained from the United States Public Health Laboratories, Washington, D. C. The bacteriophage gave characteristic plaques on agar and partial dissolution in bouillon. The color of this culture was yellow-green.

The fact that we did not succeed in isolating a bacteriophage from any of the strains isolated in our laboratories, but had no difficulty with strains transferred from other laboratories is of importance because they seemed to appear spontaneously during the cultural life of the strains. The appearance of the bacteriophage plaques when they were transferred to another laboratory implies that there may have been certain differences in the preparation of the media favoring the appearance of the bacteriophage phenomenon.

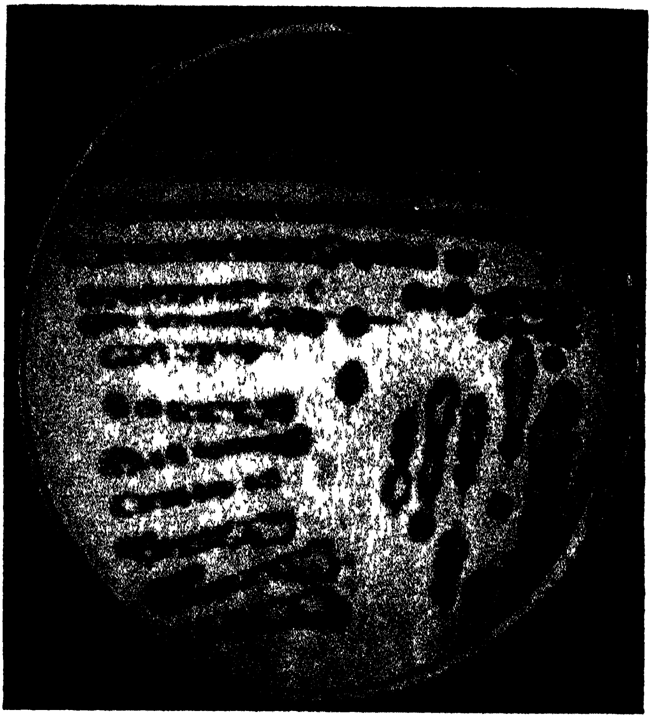

Fig. 4. Seventy-two-hour Culture of B. pyocyaneus "D" in Transmitted Light Showing Lytic Pockets, Mass and Marginal Lysis

A case of spontaneous appearance of a bacteriophage in culture of *B. pyocyaneus* after transferring to another laboratory was also reported by Gate and Gardere (1927).

The activity of the bacteriophage "I" was tested against 11 heterologous strains of *B. pyocyaneus*. Unlike the filtrates prepared from iridescent spots, this filtrate was active with almost all the heterologous strains tested.

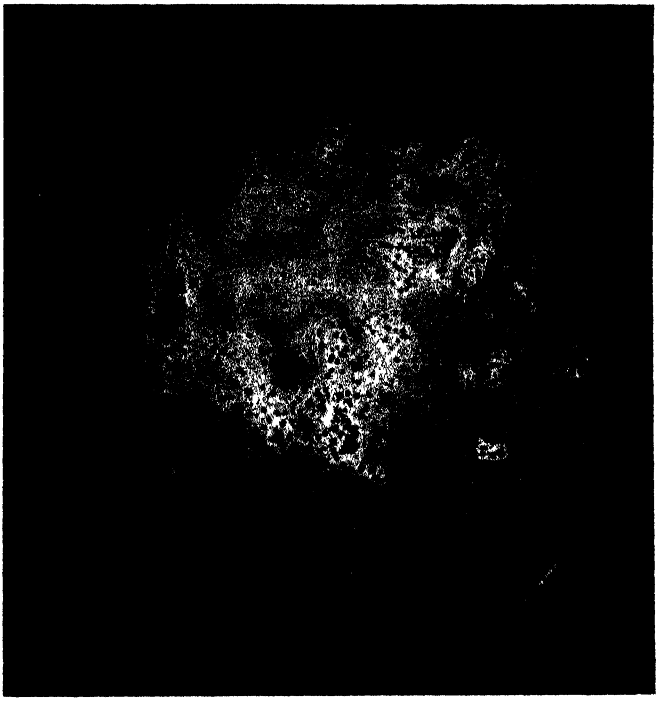

FIG. 5. SEVENTY-TWO-HOUR CULTURE OF 0.05 CC. OF A SUSPENSION OF B. PYOCYANEUS "I" PLUS 10^{3} CC. OF BACTERIOPHAGE "I"

The white mass and circular white spots represent the homogeneous culture and single isolated colonies; the dark circular rings are tasches vierges. Compare the size, appearance of these plaques with the lytic pockets in figure 4.

The activity of the anti-pyocyaneus bacteriophage was also tested with the coli-typhoid-dysentery groups. The results obtained were quite different from those of Okuda (1923) and Pesch and Sonnenschein (1925). According to these authors strains of the coli-typhoid-dysentery group were absolutely refractory to the pyocyaneus bacteriophage. The results of this experiment, recorded after 4 serial passages, repeated twice, is given in table 1. From this table it can be seen that the anti-pyocyaneus bacteriophage "I" is active with the typhoid-dysentery strains on solid and in liquid media; with *B. coli* apparently only on solid media, the bouillon tubes remaining cloudy. However, some of these cloudy cultures yielded a few plaques when transferred on agar plates.

The outstanding differences between the lytic substance that gives iridescent spots and a real bacteriophage in the sense of d'Herelle's principle may be summarized as follows:

1. The first striking difference between the lytic agent of *B. pyocyaneus* and the pyocyaneus bacteriophages is found in their morphological manifestations, namely, in the appearance of their plaques on agar plates (compare figs. 4 and 5). The lytic pockets are much larger and possess no definite form. In reflected light they exhibit characteristic iridescent lustre. They gradually extend and finally cover the surrounding media. Within the areas of the lytic spots the agar is not bare, but is covered by a transparent and blue-green vitreous layer. Under the microscope these lytic pockets reveal the presence of numerous needle-like crystals. The lytic pockets are not sterile. The bacteriophage plaques or tasches vierges of d'Herelle in the case of pyocyaneus cultures (as well as with any other cultures possessing a bacteriophage), are perfectly circular. They possess no irridescent lustre. They are not overgrown by surrounding media and may remain unchanged in their shape for weeks and even months (sometimes they are covered by secondary cultures). They are not covered with crystals. They are sterile.

2. The lytic agent of pyocyaneus cannot be separated from cultures unless it disappears after a certain number of generations. The culture possessing bacteriophage plaques can be separated from these plaques by methods of colony purification.

3. Filtrates prepared from the lytic strains of pyocyaneus cultures, tested against their homologous and heterologous strains showed silvery flecks on agar and partial lysis in bouillon with their homologous strains, but the controls which were

filtrate-free, gave exactly the same results. The filtrates of iridescent spots used as bacteriophages, did not act as such. Admixed to emulsions of lytia pyocyaneus cultures in various amounts, they did not influence the number of iridescent spots. It is therefore concluded that the filtrates of cultures with iridescent plaques do not contain the principle causing these plaques. The bacteriophage of *B. pyocyaneus* "I" tested with its homologous and 11 heterologous strains exhibits activity with all the strains, although in various degrees. The number of plaques on agar is in direct proportion in the quantities of the filtrate admixed.

CONCLUSIONS

From the above observations and experiments the following conclusions can be drawn:

Lytic agent and bacteriophages were isolated from pyocyaneus cultures, the isolation of the latter occurring under peculiar conditions. That the two principles are apparently of a different nature is shown by their characteristic morphological manifestations and biochemical reactions.

The bacteriophage of *B. pyocyaneus* "I" is active with strains of the colon-typhoid-dysentery group.

I am indebted to Mr. Francis J. Johles for the photographs used in this paper.

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SPOILAGE OF OLIVES BY COLON BACILLI[1]

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Bacterial decomposition of olives during the curing process is of considerable importance to the olive packing industry. The spoilage is characterized by the formation of shallow pockets directly under the epidermis which are filled with soft tissue detritus, brine and gas. Very often the epidermis is completely separated from the flesh and is distended so that a blister-like protuberance is formed. In badly infected olives, the entire flesh is involved. Occasionally pockets are formed that extend nearly to the pit of the fruit. These contain gas but seldom any decomposed material. The flesh is merely split and compressed sidewise, apparently by the gas pressure. Blistering of the epidermis does not occur with this splitting and usually the only external evidence of disintegration is the sponginess of the olive or a slight depression in the fruit directly above the internal gas pocket. Owing to the gas content, these olives float on the surface of the storage brines and are known as "floaters."

In early stages of infection only small, white spots under the epidermis are visible. These seem to be composed of many thread-like processes radiating into the flesh of the olive from a central point. Owing to the approximately circular appearance of these areas, the spoilage has aptly been termed "fish eye." Figures 4 to 9 in plate 1 show fresh, ripe olives typically affected by this bacterial spoilage. These were experimentally infected by purified cultures of bacteria used in this study. Figure 3 shows the crushed cells parallel to an internal gas pocket. It was found that the bacteria producing "fish eye," "floaters," and "internal gas pockets," were not different species.

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[1] This problem was begun in the Fruit Products Laboratory, College of Agriculture at the University of California, Berkeley, California. During 1933 it was studied at the Laboratory of Bacteriology, at the University of California at Los Angeles.

In general, the curing of ripe olives is divided into three stages: (1) Recently picked olives are placed in brine of varying degrees of concentration, known as "holding solutions." (2) After several weeks in "holding solution" the olives are treated intermittently with dilute NaOH and exposure to air. Finally the free NaOH is washed from the olives with water. (3) These "pickled" olives are then canned and sterilized.

The bacterial decomposition generally occurs in the "holding solution" or in the "wash" water following the final lye treatment.

Kossowicz (1908) isolated *B. coli* from green olives that were putrefying in dilute brine solutions. He found that if he replaced these olives in fresh brine solutions (2 per cent) and then initiated a lactic acid fermentation by adding sour milk the putrefaction was arrested. Heating the olives to 60° to 70°C. likewise inhibited decomposition.

Cruess and Guthier (1923) were unable to produce decomposition of sterilized olives by inoculations of *B. coli*, *B. subtilis*, or *B. vulgatus*. They were able to transmit the infection to sterilized fruit by inoculations of juice from infected olives or by the addition of disintegrating olives to sterilized fruit in weak brine solutions. A very short rod occurring singly or in pairs was isolated by them as the probable etiological factor; however, it was necessary to use this aerobe in combination with an anerobe also isolated from decomposed fruit before reproduction of typical spoilage in sterilized olives was possible. They concluded that the decomposition was caused by several types rather than a single type of bacteria.

Alvarez (1926) isolated a Gram-negative lactose-fermenting organism from olives spoiled in "wash" water, and reproduced typical decomposition in sterilized fresh olives in nutrient broth. Her organism was pleomorphic and was extremely prolific at 37°C. It withstood exposure to 80°C. for forty-five minutes although no spores were demonstrable, but was killed in twenty-four hours by a 10 per cent solution of NaCl. She concluded that the bacterium was not of the true coli group, but was of a related species and that probably more than one bacterial species could produce "floaters."

Esty (1930) found that short Gram-positive rods and yeasts were present both in normally fermented olives and in those showing gas blisters and softening. By inoculating fresh olives in 3 per cent brine, he determined that typical gas blisters were produced by certain of the purified yeast cultures within seven days at 24°C. This yeast was halophilic.

METHOD

Spoiled olives packed in samples of the original "holding solution" or "wash" water were received at the laboratory sealed in the usual olive can. In a few instances only was care taken to steam the can before collecting the samples of brine and decomposing fruit. In a number of instances, however, collections were made in sterile wide-mouthed bottles. The usual bacteriological precautions were observed for removing the spoiled material aseptically from the containers for examination.

Usually 1-ml. portions of the brine were added to tubes of glucose and lactose broth and to glucose or nutrient agar. Incubation was maintained at 32°C. for twenty-four to forty-eight hours. A loopful of broth from each tube was then streaked upon glucose or nutrient agar plates and incubated at 32°C. Colonies were picked at random from the first set of plates and planted upon agar slants. After twenty-four hours, incubation colonies were also picked from the "streak" plates and planted on agar slants.

Spoiled olives were grasped by forceps, the epidermis covering a typical decomposed area was seared with a heated needle or scalpel, and a planting needle, heated to redness, was plunged directly into the flesh. By a rotary motion of the needle, a hole was torn in the epidermis and through this a small portion of the tissue was removed and introduced into lactose and glucose broth or nutrient agar. The subsequent treatment of these tubes and plates was identical with the procedure described above.

Cultures obtained in this manner were inoculated into large test tubes of sterile nutrient broth containing 3 per cent NaCl and several sterile, fresh olives. Cultures capable of producing blisters were kept for further study. In cases of contamination, isolations were made from the experimentally spoiled fruit and the process described above repeated until the culture was purified.

EXPERIMENTAL

Induced spoilage and identification of bacteria

Cruess (1923) showed that the Sevillano and Ascolano varieties of olives are far more susceptible to bacterial spoilage than the Manzanello and Mission varieties, although any one of these types may become infected. He believed that the presence of carbohydrates and other fermentable substances within the olive tissues was the basis for this susceptibility. Nichols (1930) showed that on the dry basis the mean sugar content of the Sevillano and Ascolano varieties was 21.1 and 19.0 per cent respectively while in the Manzanello and Mission varieties it was 9.4 and 10.6 per cent respectively. For these reasons the Ascolano and Sevillano varieties were used in the present tests.

Tap water extracts of fresh olives were prepared by mincing approximately 1 kgm. of olives, softened in hot water, in 3 or 4 liters of water. This material was strained through several layers of gauze to remove the coarse particles. The solution was neutralized to approximately pH 6.8 with NaOH and 3 per cent NaCl was added. The resulting liquid had an average density of 1.009. For use as a culture medium Andrade's indicator was added.

Purified cultures of bacteria isolated from spoiled fruit and from other sources were inoculated into test tubes containing approximately 5 cc. of olive extract. Incubation was at 32°C. for seventy-two hours. From this series inoculations were made into a second series of tubes containing olive-extract medium. Usually the inoculum consisted of two or three loops per tube. In this manner each culture was passed through five different tubes of olive extract during a period of twenty-one days.

It was found that a number of the cultures capable of producing blisters in sterilized olives suspended in nutrient broth were unable to grow successfully in this extracted medium after the second transplanting. The cultures capable of successful multiplication produced a definite acidity and often showed vigorous gas production. These latter cultures were used in further tests.

Attempts to sterilize fresh olives without altering their composition or texture were unsuccessful. Autoclaving and fractional sterilization softened the flesh considerably. Chemical sterilization with NaOH or HCl removed the waxy cuticle of the olive. Mercuric chloride and tincture of iodine produced toxic fruit unless prolonged washing in sterile water was used. Olives exposed in water to 90°C. for one minute were not injured but were not always sterilized.

TABLE 1

*Spoilage of Sevillano olives suspended in olive extract medium by purified cultures**

NUMBER OF TEST	STERILIZATION OF OLIVES	EARLIEST SYMPTOMS OF SPOILAGE		PER CENT OF TUBES SPOILED AT EARLIEST SYMPTOMS		PER CENT OF TUBES SPOILED AT TEN DAYS' INCUBATION		PER CENT OF CONTROL SPOILED AT TEN DAYS' INCUBATION	
		Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
		days	*days*						
1	Fractional method	1		75		100		0	
2	Heated 90°C. in water for 1 minute	5	5	76	73	78	78	0	5†

* Incubation at 32°C.

† Doubtful bacterial spoilage.

In order to determine which cultures were etiological agents of the spoilage, the two following tests were made:

(1) Tubes containing several fresh olives suspended in olive extract medium were fractionally sterilized. Controls for sterilization were prepared. These were inoculated with 1-ml. portions of the final series of extract cultures described above. Incubation was at 32°C. for ten days.

(2) Tubes containing olive extract medium were autoclaved. To each of these were added fresh olives heated in water to 90°C. for one minute. These were then inoculated as in the above series and aerobic and anaerobic tubes prepared. Controls for each were made. Incubation was at 32°C. for ten days.

The data are given in table 1.

It was found that the cultures producing spoilage in the water-heated olives were the first to show symptoms of blisters in test 1, and that 22 per cent of the cultures forming blisters in the sterilized olives were incapable of attacking sound olives in ten days. All of these cultures, however, grew successfully in olive-extract medium. This seems to indicate that olive spoilage bacteria possess a property of invading olive tissues, and that this ability is not common to all bacteria capable of utilizing olive substances as metabolites. Test 2 likewise indicates that spoilage may occur equally well under aerobic or anaerobic conditions.

TABLE 2

Identification of spoilage bacteria by biochemical reactions

GROUPS	BIOCHEMICAL REACTIONS				
	Lactose	Dextrose	Sucrose	Salicin	Acetyl-Methyl carbinol
I	A.G.*	A.G.	A.G.	A.G.	–
II	A.G.	A.G.	A.G.	A.G.	+
III	A.G.	A.G.	–	–	–

* Acid and gas formation. Andrade indicator.

The cultures producing typical spoilage under the conditions of test 2 were considered representatives of the causative agents of bacterial decomposition in olives.

The cultures were divided by biochemical reactions into three groups. The groups are given in table 2.

These bacteria possessed several common characteristics. (1) They were Gram-negative rods. Polar bodies staining more deeply than the central portions of the cells were often found. In many instances the cells were extremely short especially in olive extract medium. (2) They were capable of fermenting lactose and glucose. (3) They grew vigorously in olive-extract medium. (4) They were non-sporulating and (5) were facultative towards oxygen. (6) Those cultures tested for cellulose digestion were found to be negative, although not every culture was studied for this property. (7) A number of these cultures were found to be killed at 55°C. in ten minutes.

SOURCE AND CLASSIFICATION OF SPOILAGE BACTERIA

Cruess (1923) was able to show that sterilized olives in 3 per cent NaCl became infected when material from the following sources was added to them: (1) fresh olives directly from trees, (2) olives (uninfected) from "holding solutions," and (3) pieces of wood or cement from infected vats. He was unable to obtain infection by addition of well water.

In the present study spoilage bacteria were successfully isolated from the following sources: (1) decomposed olives in "holding solutions" and "wash" water; (2) normal "holding solutions" containing only sound olives; (3) water supplies of several factories; (4) dust at one factory, and (5) in one instance olives picked under sterile conditions directly from a tree.

These bacteria were classified according to Bergey (1930) and were found to agree satisfactorily with the biochemical and cultural reactions of the given type species reported below.

Decomposed olives. The frequency with which spoilage bacteria were found is as follows: Group I, 70 per cent; group II, 20 per cent; group III, 10 per cent. The organisms common to both "holding solutions" and "wash" water were *E. neopolitana; E. pseudocoloides;* and *A. cloacae.* Bacteria found only in "holding solutions" were *E. gastrica,* and *A. oxytocum.* Those found in "wash" water were *E. grünthali* and *E. iliaca.*

Normal "holding solutions." Isolations were made from brine samples collected aseptically at two widely separated factories. The organisms found were *A. cloacae,* and *E. anindolica.* The latter differed from Bergey's type species in being non-motile.

Water supplies. No spoilage bacteria were found in samples from factories using city water. However, bacteria were isolated at a factory using water supplied from a ditch, and at a plant using private well water. These bacteria were found to be *E. neopolitana*, *E. iliaca*, *E. alcalescens*, and *E. pseudocoloides*. One culture of *E. iliaca* was non-motile, and one culture of *E. neopolitana* was incapable of producing indol.

Factory dust. Litmus-lactose-agar plates were exposed to the air for approximately fifteen to thirty minutes at two different packing plants. At one plant no spoilage bacteria were isolated although 70 per cent of the cultures obtained were capable of growth in olive extract medium. At the second plant, exposures were made out of doors on top of a "holding" tank. About 40 per cent of the bacteria isolated fermented lactose, and one of the cultures was capable of producing typical spoilage in olive-extract medium. This culture was identified as *E. neopolitana.* Chemically sterilized olives suspended in olive extract medium developed typical spoilage after being exposed to the air in the "pickling" room of a third factory for two weeks. These bacteria were not classified.

Miscellaneous. Samples of spoiled olives were received from a factory at which "holding solutions" tests had been made. The bacteria isolated from them were classified as *E. iliaca*, *E. neopolitana*, *E. pseudocoloidis*, *A. cloacae*, and *E. grünthali*. One culture of *E. iliaca* did not liquefy gelatin, and one culture of *E. neopolitana* did. *E. grünthali* was non-motile in olive extract medium, but was motile in nutrient broth.

The above tests indicate that bacterial decomposition of olives during the curing process is caused by a heterogeneous group of colon bacilli. These bacteria are widely spread in nature and may occur in the water supply and dust of an olive factory. Undoubtedly these latter habitats of the bacteria are sources of contamination of "holding solutions" and "wash" water. It was found that both good and spoiled tanks of olives are commonly contaminated with these bacteria. This suggests that probably tanks of olives unaffected by spoilage contain bacteriostatic substances that are absent from affected tanks.

EFFECT OF SALT AND LACTIC ACID ON SPOILAGE BACTERIA

Cruess (1923 and 1930) has studied the effects of NaCl concentrations and lactic acid fermentation on the curing of olives very thoroughly.

A lactobacillus, capable of good growth in olive extract medium, was isolated from a normal "holding solution" of Ascolano olives. This bacillus produced 0.6 per cent acid in ten days and 0.98 per cent acid in sixty days at room temperature in olive extract medium enriched with 0.5 per cent glucose. Classification according to Bergey (1930) indicated that it was closely related to *Lactobacillus beijerincki* although differing from the type species in that it was incapable of acidifying milk.

The antagonistic action of this lactobacillus to spoilage bacteria was determined. Two flasks containing 100 cc. of sterile olive-extract medium enriched with 25 per cent nutrient broth and 0.5 per cent glucose were inoculated with 0.5-cc. portions of a lactobacillus culture. An olive-extract culture of *E. neopolitana* was then added to one of these flasks in a 0.5-cc. amount and a similar culture and amount of *A. cloacae* was added to the other.

Titration for acidity in grams of lactic acid per 100 cc. of medium were made with phenolphthalein as the indicator. Portions of 0.1 cc. were immediately removed from these flasks and inoculated into lactose and glucose broth tubes which were incubated at 32°C. The tubes were read for acid or acid and gas production after twenty-four hours. Three other identical flasks were prepared, each inoculated with one of the above three cultures as controls. These were treated exactly as were the two test flasks.

After forty-eight hours' incubation, the titrations were repeated and 0.1-cc. samples were removed from the flasks for inoculation into tubes or glucose and lactose broth. Gas production in lactose and glucose broth indicated survival of the spoilage bacteria. Acid production in glucose broth without gas indicated survival of the lactobacillus. The data are shown in table 3.

These data show that acid production by the lactobacillus was not inhibited in the presence of spoilage bacteria. On the other hand, the spoilage bacteria were rapidly killed by the lactobacillus. *E. neopolitana* was unaffected by 0.38 per cent lactic acid (pH 4.2); was prevented from utilizing lactose by 0.5 per cent acid; and was killed by 0.6 per cent acid. This required six days. *A. cloacae* apparently was killed by 0.5 per cent acid on the fourth day.

TABLE 3

Antagonistic action of lactobacillus and spoilage bacteria

INCUBATION AT 32°C.	CULTURES							
	E. neopolitana				*A. cloacae*			
	Acidity†	pH	Lactose	Dextrose	Acidity	pH	Lactose	Dextrose
days								
0	0.0072	6.3	A.G.*	A.G.	0.0072	6.3	A.G.	A.G.
2	0.0386	4.2	A.	A.G.	0.0480	4	A.G.	A.G.
4	0.0504	3.7	—	A.G.	0.0570	3.7	—	A.
6	0.0594	3.7	—	—	0.0648	3.7	—	—
				A in 48 hours				A. in 48 hours
8	0.0612	3.7	—	—	0.0648	3.7	—	A.

INCUBATION AT 32°C.	CONTROLS								
	E. neopolitana			*A. cloacae*			Lactobacillus		
	Acidity	Lactose	Dextrose	Acidity	Lactose	Dextrose	Acidity	Lactose	Dextrose
days									
0	0.0072	A.G.	A.G.	0.0072	A.G.	A.G.	0.0072	-	A.
2		A.G.	A.G.		A.G.	A.G.		—	A.
4		A.G.	A.G.		A.G.	A.G.		—	A.
6		A.G.	A.G.		A.G.	A.G.		—	A.
8	0.010	A.G.	A.G.	0.0090	A.G.	A.G.	0.0594	—	A.

* A.G. = acid and gas formation.

† Grams of lactic acid per 10 cc. mediums.

To determine the lethal action of lactic acid, tubes of enriched olive-extract medium containing lactic acid (by weight) were inoculated with spoilage bacteria and the lactobacillus. A loopful from each test tube was then streaked onto a glucose agar plate. These were incubated at 32°C. for forty-eight hours before reading. This procedure was again repeated after twenty-four hours. The data are shown in table 4.

E. iliaca survived 0.25 per cent lactic acid for twenty-four hours but was killed in forty-eight hours. *E. neopolitana* and *A. cloacae* showed very feeble resistance to 0.25 per cent acid in twenty-four hours. The lactobacillus was not affected by 1.0 per cent acid in this length of time. It is interesting to note that the germicidal concentrations of lactic acid vary in tables 3 and 4. This is probably because the acidities recorded in table 3 represent not only lactic acid but also other by-products of bacterial metabolism; however it is possible that an "acquired" tolerance is produced in spoilage bacteria which have been grown in the presence of the lactobacillus. This has not been tested.

TABLE 4

Lethal action of lactic acid

	CONCENTRATION IN PER CENT														
	1			0.75			0.5			0.25			0.1		
	Time of exposure in hours														
	0	24	48	0	24	48	0	24	48	0	24	48	0	24	48
E. iliaca	+*	−	−	+	−	−	+	−	−	+	+	−	+	+	+
E. neopolitana	+	−	−	+	−	−	+	−	−	+	±†	−	+	+	+
A. cloacae	+	−	−	+	−	−	+	−	−	+	±	−	+	+	+
Lactobacillus	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+

* + = growth of bacteria.

† ± = slight growth.

The bacteriostatic concentration of lactic acid was found to parallel closely the germicidal concentration. In this test, *E. iliaca, E. neopolitana*, and *A. cloacae* grew very slowly at room temperature in enriched olive-extract agar containing 0.1 per cent lactic acid, but not in media containing higher concentrations of acid. The control cultures, however, grew rapidly, causing vigorous splitting of the agar.

These tests show that lactic acid is definitely germicidal to spoilage bacteria, and indicate that lactobacilli in "holding solutions" are important factors in preventing spoilage. Likewise these tests indicate that solutions with acidities between 0.3 and 0.5 per cent may contain viable spoilage bacteria.

To determine the lethal action of NaCl on the spoilage organisms and the lactobacillus, tubes of enriched olive extract medium containing varying concentrations of NaCl were inoculated with *E. neopolitana*, *E. iliaca*, *A. cloacae*, and *Lactobacillus* sp. Two series, one at pH 5 and one at pH 8.5 were prepared for each culture. Incubation was made at room temperature. Test streaks were made onto glucose agar plates.

It was found that each organism survived a concentration of 20 per cent NaCl in twenty-four hours. At pH 8.5 *E. iliaca* was killed by 15 per cent NaCl in forty-eight hours while *E. neopolitana* and *A. cloacae* were killed by 18 and 19 per cent respectively. However each of these bacteria survived 19 per cent NaCl for forty-eight hours at pH 5.0. These results corresponded with those obtained with *E. anindolica* in glucose broth. At pH 8.5 disinfection occurred at 13 per cent NaCl and at pH 5.0 at 18 per cent in forty-eight hours. The lactobacillus survived a concentration of 19 per cent NaCl at pH 8.5 and 20 per cent at pH 5.0 in forty-eight hours.

TABLE 5

Bacteriostatic action of NaCl on spoilage bacteria

CULTURES TESTED	pH OF MEDIUM	CONCENTRATION				
		8 per cent	7 per cent	6 per cent	5 per cent	Control
E. iliaca *E. neopolitana* *A. cloacae*	5.0	–	–	A.G.	A.G.	A.G.
	8.5	–	A.G.	A.G.	A.G.	A.G.

The bacteriostatic concentration of NaCl was found to be very much lower, however. This test was conducted exactly as with lactic acid. The data are given in table 5.

In order to obtain the bacteriostatic concentration of NaCl for the lactobacillus flasks with 100 cc. of enriched olive-extract medium containing varying amounts of salt were seeded with 0.5 cc. of an olive-extract culture of *Lactobacillus* sp. Titrations for grams of lactic acid per 10 cc. were made immediately. The flasks were then incubated at room temperature for forty-eight hours when the acidity was again determined. Two series of these flasks were prepared, one at pH 5.0 and one at pH 8.5. Since acid production was extremely low in the basic series the data are omitted from table 6.

These tests show that relatively low concentrations of NaCl are bacteriostatic to the lactobacillus and spoilage bacteria in an acid medium. This agrees with Winslow and Dolloff (1928) who found that 1 molar NaCl was highly toxic for *B. coli* in tartrate medium but did not sterilize the solution in twenty hours. Likewise these tests show that very high concentrations of NaCl are necessary to exert a rapid germicidal action upon the microörganisms. This agrees with Hotchkiss (1923) who found that a 1 per cent peptone solution containing 2 molar NaCl inhibited growth of *B. coli*. It is also shown that the lethal action of NaCl is more effective in a basic medium than in an acidic one but that the reverse is true, to a slight degree, as to bacteriostatic effect.

TABLE 6

Bacteriostatic action of NaCl on lactobacillus

INCUBATION AT 32°C.	pH OF MEDIUM	CONCENTRATION IN PER CENT						
		10	9	8	7	6	5	0
		Acidity in grams lactic acid per 10 cc.						
days								
0	5.0	0.0072	0.0072	0.0072	0.0072	0.0072	0.0072	0.0072
1		0.0072	0.0072	0.0081	0.0072	0.0081	0.0072	0.0153
2		0.0072	0.0072	0.0072	0.0072	0.0081	0.0099	0.0288
4		0.0072	0.0081	0.0081	0.0117	0.0135	0.0171	0.0450
6		0.0072	0.0081	0.0081	0.0135	0.0153	0.0198	0.0522

This indicates that NaCl is not an important factor in preventing olive spoilage in "holding solutions" since the concentration of NaCl necessary to inhibit growth of spoilage bacteria is beyond the limit packers usually care to utilize.

SUMMARY AND CONCLUSIONS

1. It has been shown that bacterial spoilage of ripe olives in "holding solutions" and "wash" water is caused by bacteria of the genera Escherichia and Aerobacter.
2. The bacteria were found as common contaminants of the "holding solutions" and in factory dust. Also they were observed as contaminants of the water supply of two factories.
3. These colon bacteria were markedly inhibited by growth of a lactobacillus isolated from a normal "holding solution." Olive-extract medium containing heavy inoculums of spoilage bacteria was sterilized within four to six days by this lactobacillus at 32°C. It was also determined that 0.25 per cent lactic acid was germicidal for spoilage bacteria in twenty-four hours.
4. These colon bacilli were resistant to 20 per cent NaCl for twenty-four hours, but were killed by 19 to 15 per cent in forty-eight hours. The lethal action was increased in a basic medium. A 7 per cent concentration of NaCl in an acidic medium was found to be bacteriostatic to the microörganisms.
5. It is concluded that lactobacilli are the natural agents controlling bacterial spoilage of olives in "holding solutions."

Sincere thanks are extended to Dr. W. V. Cruess for the suggestion of this problem and for his valuable aid during its study. Many thanks are due Dr. T. D. Beckwith for his helpful advice and criticisms. Gratitude is expressed to Elizabeth E. Phillips for histological preparations and to Boris Krichesky and Taki A. Shima for photographs.

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PLATE 1

Fig. 1. Tissue of a normal, sound olive, Sevillano variety, showing cuticle covering the regular, flattened epidermal cells. × 100. Mallory hematoxylin and light green stain.

Fig. 2. Tissue of a typically infected olive showing intact cuticle and heavily infected epidermal and parenchymal cells. The epidermis is still attached to the flesh. Note that the infection radiates from the central point of decomposition along the epidermis. The bacteria stained with hematoxylin and the tissue with light green. Natural infection in olive from "wash" water. × 100.

Fig. 3 Tissue of a typically infected olive with gas pockets. The parenchymal cells are crushed, but do not appear disintegrated. See figure 9. This section is well below the epidermis and parallel to the internal gas pocket. Bacteria were found scattered throughout cells surrounding area when examined under oil immersion. Experimental infection. × 100. Mallory hematoxylin and light green stain.

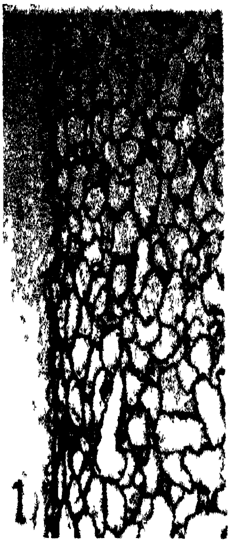
Fig. 4. Olive showing incipient "fish eye" spots. Experimental infection. Natural size.

Fig. 5. Large "fish eye" spots showing radiating lines of infection from central point of decomposition Experimental infection. Natural size.

Fig. 6. Typical "floater" with epidermis loosened from flesh. Note the extensive area involved. Experimental infection. Natural size.

Fig. 7. Blister formation showing a small gas bubble under epidermis. Experimental infection. Natural size.

Fig. 8 and 9. Typical "floater" with internal gas pockets. Note signs of depressed epidermis in figure 8. No disintegration is visible. Experimental infection. Natural size.



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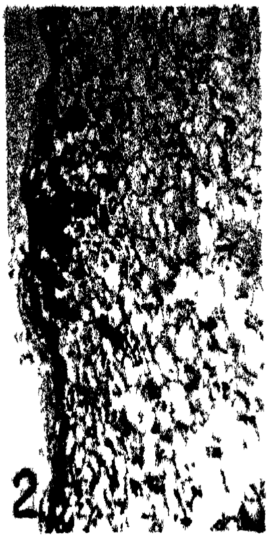

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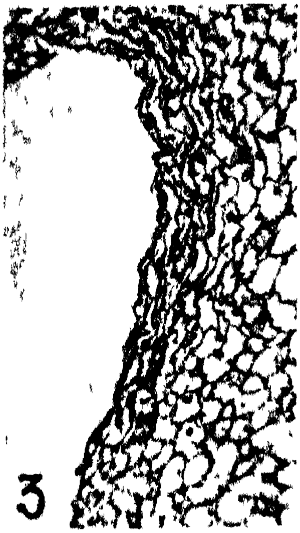

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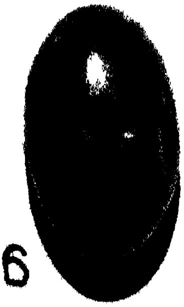

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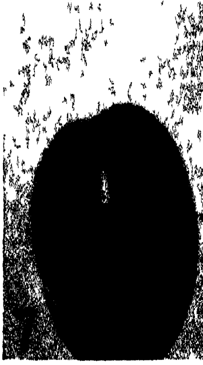

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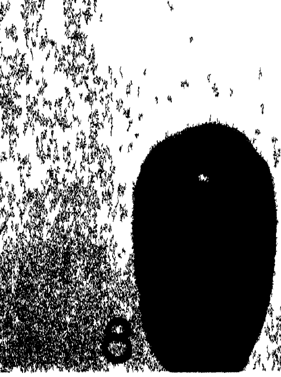

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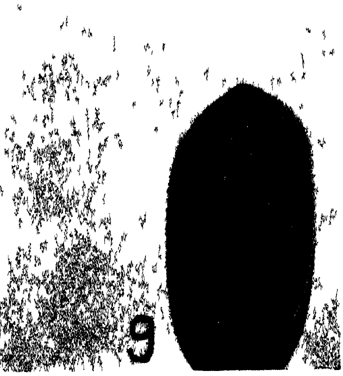

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(Ralph L. Tracy: Spoilage of olives by colon bacilli)

STUDIES ON ANAEROBIC BACTERIA

I. A CORN-LIVER MEDIUM FOR THE DETECTION AND DILUTION COUNTS OF VARIOUS ANAEROBES[1]

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In the cultivation of the thermophilic anaerobic organisms causing "swells" in canned foods[2] difficulty was encountered in finding a suitable medium for routine cultivation. Of the usual media employed for anaerobes, liver infusion is most satisfactory for growth, but has the disadvantage of requiring some anaerobic seal or incubation in an anaerobic jar for best results. When large numbers of tubes are to be used, the time necessary for sealing becomes a limiting factor, and incubation in anaerobic jars is impractical. Semi-solid media avoid in part the necessity of sealing the inoculated tubes; corn mash (4 to 8 per cent) is a medium of this type and has been much used for anaerobes of the nitrogen-fixing butyric type. For the thermophilic anaerobes plain corn mash was not found to be a suitable medium, and, in order to enrich the medium for this group, dried liver was added. Preliminary tests with this combination showed it to be more suitable than plain corn mash.

The medium is made from ordinary whole corn meal, dried liver and water. The 1 to 2 per cent liver (tissue from liver-infusion medium dried at 55° to 60°C. and finely ground) and 5 per cent corn meal are steamed one hour, cooled and tubed. The resulting, rather viscous, medium requires care in sterilizing; this may be accomplished by autoclaving for two hours at 15 to 17 pounds. If pressure is reduced slowly after sterilization, short

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[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

[2] The taxonomy of these cultures will be presented in a later paper of this series.

(6-inch) tubes may be used without blowing the plugs. This medium need not be steamed just before using, requires no seal, nor incubation in an anaerobic jar, and satisfactory results may be obtained with two to five centimeters depth of medium.

If this medium is to prove useful for anaerobic organisms in general, it must possess, in our opinion, a certain combination of advantages. Some of these are availability of materials, ease of preparation and sterilization, and low cost of ingredients if the medium is to be used in large quantities. In addition, a good medium should support growth of as many representatives as possible of the group for which it is intended; differential reactions for members within the group are also desirable. For use in routine detection, positive results should be obtained in the minimum of time, and in all cases growth from small inocula is desirable. Furthermore, readings of positive growth should be of such nature as to be constant and easily detected. And lastly, for spore-forming organisms the ability of the medium to allow sporulation is of convenience in maintenance of stock cultures. We believe that the corn-liver medium described above more nearly fulfills these requirements for the various groups of anaerobic bacteria then any other medium with which we have worked.

Certainly the critical test of growth in a medium is not the result obtained by the use of a heavy inoculum, such as 1 cc. or more of an active culture, but preferably that of initiation of growth from a low concentration of viable cells. In order to test the efficiency of the corn-liver medium, it was compared on this basis with a number of other media generally used for anaerobes. Those chosen were litmus milk with reduced iron (Hastings and McCoy, 1932), brain (Hall, 1920), Robertson's alkaline beef heart (Levine and Schoenlein, 1930), liver infusion[3] (each tube

[3] Modified formula of Cameron and Williams (1928): Steam 500 grams of ground beef liver (from which the fat has been removed) with 1000 cc. of tap water for two to three hours. Cool and strain through cheese cloth. Make filtrate to original volume and add 1 per cent Bacto-peptone and 0.1 per cent K_2HPO_4. Sterilize filtrate in stock flasks (thirty minutes at 15 pounds); dry the tissue at 55° to 60°C. When the medium is needed, tube the infusion prepared above over small chunks of the dried tissue and resterilize as above.

TABLE 1

*Comparison of media with respect to growth initiation of dilute inocula of spore-forming anaerobes**

CULTURE		ALKALINE EGG			MILK, Fe			BRAIN			BEEF HEART			LIVER			CORN			CORN, Fe			CORN LIVE	
		a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b
Thermophile 3	1000	—	—	—	—	1	3	2	1	1′	—	—	—	2	3	3	1	1	—	—	—	2	2	2
	100 T	—	—	—	—	—	3	—	1	1′	—	—	—	—	3	3	1	1	1	—	—	2	2	2
	1 M	—	—	—	—	1	3	—	—	1′	—	—	—	—	2	3	—	1	1′	—	—	2	1′	2
	10 M	—	—	—	—	—	1′	—	—	—	—	—	—	—	—	3	—	—	—	—	—	2′	—	2
Thermophile 7	1000	—	—	—	1	1	3	1′	1	1	—	—	—	1	3	3	1	2	1	—	—	2	2	3
	100 T	—	—	—	—	1′	3	—	—	—	—	—	—	—	2	3	—	1	1	—	—	2	—	3
	1 M	—	—	—	—	1	3	—	—	1	—	—	—	—	1	3	—	1	1′	—	—	2′	—	2
	10 M	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—	—	1	—	—	2′	—	2′
Thermophile 12	1000	—	—	—	1	1′	3	2	—	—	—	—	—	3	3	3	1	1	1	1′	—	2	2	2
	100 T	—	—	—	—	1	3	—	1	—	—	—	—	1	3	3	1′	1′	1	—	—	2	2	2
	1 M	—	—	—	—	1	3	—	1	—	—	—	—	—	2	3	—	1	1	—	1′	2	—	2
	10 M	—	—	—	1′	1′	3	—	—	—	—	—	—	—	—	3	—	—	1′	—	—	2′	—	1′
Butyric 26	1000	—	—	—	1	2	3	1	1	—	—	—	—	1	3	3	1	2	3	1′	2	2	3	3
	10 T	—	—	—	—	1	3	—	1	—	—	—	—	—	3	3	—	2	3	—	1	2′	3	3
	100 T	—	—	—	—	—	3	—	1′	—	—	—	—	—	2	3	—	1	3′	—	—	2′	—	3
	1 M	—	—	—	—	—	3′	—	—	—	—	—	—	—	1	3′	—	—	3′	—	—	—	—	3′
Butyric 37	1000	—	—	—	1	2	3	1′	1′	—	—	—	—	1′	3′	3	3	3	3	3	3	3	3′	3
	10 T	—	—	—	1′	2	3	—	1′	—	—	—	—	1	2	2	2	2	2	1′	2′	2′	3	3
	100 T	—	—	—	—	1	3′	—	—	—	—	—	—	—	1	3′	2′	2′	2′	—	—	2′	—	—
	1 M	—	—	—	—	1	3′	—	—	—	—	—	—	1	1	1	—	—	—	—	—	—	3′	3′
Butyric 40	1000	—	—	—	1	1	3	—	—	—	—	—	—	3	3	3	1	2	3	2	2	3	3	3
	10 T	—	—	—	—	—	3	—	—	—	—	—	—	1′	3	3	1	1	3	—	1	3	3	3
	100 T	—	—	—	—	—	3	—	—	—	—	—	—	—	3	3	1′	2	3	—	2	3	3	3
	1 M	—	—	—	—	—	3	—	—	—	—	—	—	—	3	3	1′	1′	3′	—	2′	3	3	3
Cl. pasteurianum	1000	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—	—	1	—	—	—	—	1
	10 T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—	—	1′	—	—	1	—	—
	100 T	—	—	—	—	—	1′	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	1 M	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Cl. felsineum	1000	—	—	—	—	1	3	—	—	—	—	—	—	—	—	3	—	1	3	—	—	3	—	3
	10 T	—	—	—	—	—	3	—	—	—	—	—	—	—	—	3	—	1	3	—	—	3′	1	3
	100 T	—	—	—	—	—	3	—	—	—	—	—	—	—	1	3	—	2	3	—	—	3	3	3
	1 M	—	—	—	—	—	3′	—	—	—	—	—	—	—	—	2′	—	—	3	—	—	—	—	2
Cl. multifermentans	1000	—	—	—	2	3	3	1	1	—	—	1	1	3	3	3	1	2	2	—	—	—	3	3
	10 T	—	—	—	2	2	3	—	1	—	—	1′	—	3	3	3	1′	2	2	—	—	1′	3	3
	100 T	—	—	—	—	1′	3	1′	1	—	—	—	—	1′	3′	3	1	2′	2′	—	—	—	3	3
	1 M	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* The dilution figures represent the dilution of the inoculum ordinarily given one tube.

The columns "a, b, c" in every case represent the readings taken following incubation of 18, 24 and 120 hours respectively.

The figures "1, 2, 3" represent progressive stages of growth of the culture in the medium in question; — means no visible growth. Each figure on the table represents the reading for triplicate tubes which showed approximately the same stage of growth, unless the figure is followed by a "′" mark. In such case the triplicate tubes were not in agreement; then the highest reading is given.

TABLE 1—*Concluded*

CULTURE		ALKALINE EGG			MILK, Fe			BRAIN			BEEF HEART			LIVER			CORN			CORN, Fe			CORN LIVE	
		a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b
Cl. acetobutylicum	1000	—	—	—	1	1	3	—	—	—	—	—	—	3	3	3	2	2	3	—	—	3	3	3
	10 T	—	—	—	—	1′	3	—	1′	—	—	—	—	3	3	3	1	3	3	—	—	3	3	3
	100 T	—	—	—	—	1	3	—	1	—	—	—	—	1	3	3	—	2	3	—	—	2	3	3
	1 M	—	—	—	—	—	3	—	—	—	—	—	—	—	3	3	—	2	3	—	—	3′	3	3
Cl. botulinum (type B)	100	—	—	—	—	1	3	—	—	1	—	—	3	2	3	3	—	—	—	—	—	—	1	1
	1000	—	—	—	—	—	—	—	—	1	—	—	3	2	3	3	—	—	—	—	—	—	1	1
	10 T	—	—	—	—	—	—	—	—	—	—	—	2	2	3	3	—	—	—	—	—	1	1	1
	100 T	—	—	—	—	—	—	—	—	—	—	—	3	2	3	3	—	—	—	—	—	—	1	1
Cl. oedematiens	100	—	—	—	—	1	3	—	1′	—	1	1	3	2	2	2	—	—	—	—	—	1	2	2
	1000	—	—	—	—	1	3	1	—	1	—	1′	3	2	3	3	—	—	—	—	—	1	1	2
	10 T	—	—	—	1	1	3	—	—	1	—	—	3	1	3	2	—	—	—	—	—	1	1	2
	100 T	—	—	—	—	—	—	—	1′	—	—	—	3	1	2	2	—	—	—	—	—	—	—	2
Cl. tyrosinogenes	100	—	—	—	1	1	3	—	—	2	—	—	3	1	3	3	—	—	—	—	—	—	1	1
	1000	—	—	—	—	1	3′	—	—	—	—	—	3	1	—	3	—	—	—	—	—	—	—	1
	10 T	—	—	—	—	—	—	—	—	—	—	—	1	—	—	3	—	—	—	—	—	—	—	—
	100 T	—	—	—	—	—	3	—	—	—	—	—	3	—	—	3	—	—	—	—	—	—	—	—

sealed with a layer of sterile agar after inoculation), alkaline egg (Robertson, 1931), plain corn mash, with and without the addition of reduced iron, and corn-liver mash. *Cl. botulinum* (types A, B, and C), *Cl. oedematiens*, *Cl. sporogenes*, *Cl. tyrosinogenes*, *Cl. acetobutylicum* (three strains), *Cl. pasteurianum*, *Cl. felsineum*, *Cl. multifermentans*, eight strains of unidentified butyric anaerobes of soil origin, and five strains of thermophilic anaerobes were chosen as representative test organisms to be studied in this comparison. Dilutions from suspensions of spores were made in sterile tap water in the usual manner, and 1 cc. inocula were introduced into triplicate tubes of the various media. Readings were taken at the end of eighteen, twenty-four and one hundred twenty hours of incubation at the appropriate temperature.

Of the twenty-five organisms tested in this manner, thirteen are represented in table 1; they are of widely different types, and their reactions may be taken as typical of the results obtained. It is clear that certain of the media are distinctly inferior as, for example, alkaline egg, which failed to support growth of any of the organisms in this experiment with the inocula used. Beef heart is satisfactory for pathogenic anaerobes, but not for the thermophilic and soil butyric types. Conversely, plain corn and corn with reduced iron are very satisfactory for the soil butyrics, and allow fair growth of the thermophiles, but are practically useless for the pathogens. Brain, although widely used for pathogenic anaerobes, gave rather erratic results in this experiment. It did allow growth of several types of anaerobes, but the readings were indefinite. The readings in the table are based upon evidence of gas or turbidity. In this medium gassing is often transient, as in the case of the butyrics, and when there is no evidence of blackening or decomposition of tissue, readings based upon gas production are unreliable. Reference to the table will show several examples of inconsistent readings in this medium. The milk with reduced iron may also be disqualified, as it will be noted that in most cases positive growth did not occur early and, in some cases, not at all in the higher dilutions of the inoculum as, for example, *Cl. botulinum* type B.

Thus, it may be seen that the superior media, as shown by these results, are liver infusion and corn-liver mash. Both appear equally good from the standpoint of the dilution of inoculum which gave growth within the total time of the test, although the corn-liver medium is somewhat better for early readings. In addition, the liver infusion possesses some of the disadvantages mentioned above; it is more difficult to prepare and inoculate than corn-liver medium.

In a shorter series of tests similar to the one above, the relative value of the new medium, tryptone egg recently recommended by Spray (1933), was tested. It was found to be far inferior to the corn-liver medium as a medium for all types of anaerobes, allowing growth chiefly of the proteolytic types.

It is well known that consistent counts by the dilution method are difficult to obtain with anaerobes. In order to test further the qualities of the corn-liver medium, a series of dilution counts of both pure cultures and natural mixtures, such as are found in lake mud, soil, and silage, was made. In this experiment three media were used. Winogradsky's nitrogen-free solution (1902), Ritter's glucose peptone agar (1932), and corn-liver mash. The first two media were chosen because they had formerly been used for similar counts and because they should be selective for the anaerobic flora of the natural mixtures used in this test.

TABLE 2

*Dilution counts of anaerobic spores in pure cultures and in natural mixtures**

INOCULUM	DILUTION	WINOGRADSKY'S SOLUTION			RITTER'S GLUCOSE PEPTONE AGAR			CORN-LIVER MASH		
		a	b	c	a	b	c	a	b	c
Clostridium pasteurianum	10 T	—	—	—	—	—	—	—	—	3
	100 T	—	—	—	—	—	—	—	—	—
Clostridium acetobutylicum	10 T	—	—	—	—	—	3	2	3	3
	100 T	—	—	—	—	—	3	—	2	3
	1 M	—	—	—	—	—	3	—	1	3
	10 M	—	—	—	—	—	—	—	—	3
	100 M	—	—	—	—	—	—	—	—	—
Butyric no. 45	10 T	—	—	—	—	—	—	2	3	3
	100 T	—	—	—	—	—	—	2	3	3
	1 M	—	—	—	—	—	—	—	—	—
Soil "A"	10 T	—	—	—	—	—	3	1	1	3
	100 T	—	—	—	—	—	—	—	—	3
	1 M	—	—	—	—	—	—	—	—	—
Silage	100	—	—	—				1	2	2
	1000	—	—	—				—	—	2
	10 T	—	—	—				—	—	2
	100 T	—	—	—				—	—	2
	1 M	—	—	—				—	—	—
Lake mud	10 T	—	—	—	—	—	—	2	3	3
	100 T	—	—	—	—	—	3	2	2	3
	1 M	—	—	—	—	—	—	—	—	1
	10 M	—	—	—	—	—	—	—	—	—

* All symbols are the same as in Table 1.

The pure cultures included were *Cl. pasteurianum*, *Cl. acetobutylicum*, and a typical butyric organism of the starch-hydrolyzing group. These cultures are representative of the types to be expected in the dilution counts of the natural mixtures. The results are summarized in table 2. The superiority of the corn-liver medium is obvious, both as regards early appearance of growth and initiation of growth in high dilutions.

TABLE 3

*Dilution counts of the vegetative cells of various anaerobes**

INOCULUM	DILUTION	WINOGRADSKY'S SOLUTION			RITTER'S GLUCOSE PEPTONE AGAR			CORN-LIVER MASH		
		a	b	c	a	b	c	a	b	c
Clostridium acetobutylicum (strain W)	10 T	—	—	—	—	—	—	2	3	3
	100 T	—	—	—	—	—	—	—	3	3
	1 M	—	—	—	—	—	—	—	2	3
	10 M	—	—	—	—	—	—	—	—	—
Clostridium acetobutylicum (strain C)	10 T	—	—	—	—	—	3	—	2	3
	100 T	—	—	—	—	—	3	—	2	3
	1 M	—	—	—	—	—	—	—	—	3
	10 M	—	—	—	—	—	—	—	—	3
	100 M	—	—	—	—	—	—	—	—	—
Clostridium pasteurianum	10 T	—	—	—	—	—	3	2	2	2
	100 T	—	—	—	—	—	3	2	2	2
	1 M	—	—	—	—	—	—	1	2	2
	10 M	—	—	—	—	—	—	—	1	2
	100 M	—	—	—	—	—	—	—	—	—
Butyric no. 45	10 T	—	—	—	—	3	3	3	3	3
	100 T	—	—	—	—	1	3	2	3	3
	1 M	—	—	—	—	—	—	2	3	3
	10 M	—	—	—	—	—	—	2	3	3
	100 M	—	—	—	—	—	—	1	2	3
	1 B	—	—	—	—	—	—	—	—	—
Thermophile no. 12	1000	—	—	—				1	2	3
	10 T	—	—	—				—	—	3
	100 T	—	—	—				—	—	3
	1 M	—	—	—				—	—	3
	10 M	—	—	—				—	—	—

* All symbols are the same as in Table 1.

Even more difficult to obtain are consistent dilution counts of vegetative cells of anaerobic bacteria. Table 3 gives the summary of a series, similar to the one above, in which only vegetative inocula were used. Dilutions here were prepared in the usual manner, with vigorous shaking. Again, the superiority of the corn-liver medium is manifest. A striking example of this is seen in the comparative counts with the cells of Butyric no. 45 and *Cl. pasteurianum.*

In this laboratory a wide variety of uses have been found for this medium. To the above types of experiments, which have been extensively used in other problems, may be added the detection of anaerobes in class studies of spoiled canned foods and chocolate candies, and for subculturing the survivors of thermal death time studies. Equal success has been met with in these instances.

There is one possible limitation to the use of the corn-liver medium, of which the authors are well aware but do not consider as detracting seriously from its value. During drying of the liver tissue in the manner noted (at 55°–60°C. for a variable time, depending upon the mass of tissue) there occurs a growth of microorganisms upon the liver. These cells are peculiarly resistant and appear in the finished medium as readily stainable gram-positive rods, granular and spore-forming. They are not viable after the sterilization suggested, as was proved by numerous attempts at sub-culture and incubation tests of uninoculated sterilized media at mesophilic and thermophilic temperatures. They do not then have any significance in uses involving cultural reactions upon the corn-liver medium. One might suppose that in microscopic work, as in enrichment of anaerobes from an unknown source, they might prove annoying. However, at least in liver dried under our conditions, the form developing is so characteristic that one can easily learn to recognize it and not be confused. The amount of contamination varies from batch to batch of liver; it may be that a way can be found to avoid its development altogether.

The following anaerobes have been cultured in this medium with success: thermophilic anaerobes causing "swells" in canned foods (25 strains), certain butyric anaerobes of soil origin—*Cl. pasteurianum* type (6 strains) and *Cl. saccharobutyricum* type (37 strains), *Cl. acetobutylicum* (19 strains), *Cl. felsineum*, *Cl.*

TABLE 4

Summary of the reactions of various anaerobes in corn-liver medium

ORGANISM	SOURCE	REACTION		
		Gas	Hydrolysis of starch	"Head" formation*
Thermophilic anaerobes (25 strains)	Isolated from various sources (spoiled canned foods, sugar, soil)	++	+	–
Butyric anaerobes of soil origin:				
Cl. pasteurianum type (6 strains)	Isolated from soil	++	–	–
Cl. saccharobutyricum type (37 strains)	Isolated from soil	+++	++	–
Cl. acetobutylicum (19 strains)	Various sources	+++	+++	+++
Cl. felsineum	A. J. Kluyver strain	+++	+++	+++
Cl. multifermentans	Amer. Type Culture No. 3538	+++	++	–
Cl. multifermentan stenalbus	Lister Institute No. 2032	++	++	–
Cl. tertium	Lister Institute No. 336	++	–	–
Cl. bifermentans	Lister Institute No. 506	++	–	–
Cl. fallax	I. C. Hall No. 254	++	–	–
Cl. aerofoetidum	I. C. Hall No. 6685	++	–	–
Cl. sphenoides	I. C. Hall No. 146	++	–	–
Cl. centrosporogenes	I. C. Hall No. 76	++	–	–
Cl. tetanomorphum	I. C. Hall No. 2992	++	–	–
Cl. histolyticum	I. C. Hall No. 1091	++	–	–
Cl. chauvoei (2 strains)	I. C. Hall No. 6	++	–	–
	Univ. of Texas	++	–	–
Cl. botulinum:				
Type A	Amer. Type Culture No. 442	++	–	–
Type B	Amer. Type Culture No. 437	++	–	–
Type C	Amer. Type Culture No. 438	++	–	–
Cl. oedematiens	Univ. of Wis. Med. School	++	–	–
Cl. sporogenes	Amer. Type Culture No. 459	++	++	–
Cl. putrefaciens	Amer. Type Culture No. 4190	+	–	–
Cl. putrificum	Amer. Type Culture No. 3559	+	–	–
Cl. tetani	Amer. Type Culture No. 4260	++	–	–
Cl. welchii	Amer. Type Culture No. 3624	++	++	–
Cl. oedematis-maligni	Univ. of Texas	++	–	–
Cl. tyrosinogenes	Amer. Type Culture No. 690	++	–	–

* The head is the mass of insoluble matter raised to the surface of the medium by virtue of the slime production of the culture and consequent trapping of the gas.

multifermentans, *Cl. multifermentans tenalbus*, *Cl. tertium*, *Cl. bifermentans*, *Cl. fallax*, *Cl. aerofoetidum*, *Cl. sphenoides*, *Cl. centrosporogenes*, *Cl. tetanomorphum*, *Cl. chauvoei* (2 strains), *Cl. botulinum* (types A, B and C), *Cl. oedematiens*, *Cl. sporogenes*, *Cl. histolyticum*, *Cl. putrefaciens*, *Cl. putrificum*, *Cl. tetani*, *Cl. welchii*, *Cl. oedematis-maligni*, and *Cl. tyrosinogenes*. Spore formation has been noted in this medium in all of the cultures, with the possible exception of *Cl. fallax*. Various types of reaction produced in this medium are shown in table 4. As the corn-meal provides a high starch-containing medium, upon which starch hydrolysis may be detected by clearing and liquefaction of the mash, this is the most striking differential characteristic. As may be seen from the table, hydrolysis of starch is a common feature among the soil butyrics, but rarely occurs in the pathogenic group. Nevertheless, gas formation was observed in many of the cultures which do not attack starch. This is presumably due to the fermentation of soluble carbohydrates of the corn-meal. In certain cases there is differentiation within the starch-fermenting group, e.g., *Cl. acetobutylicum vs. Cl. saccharobutyricum* type. Among the non-starch-fermenting group there is no marked differentiation. Although the above cultural characteristics are recognizable, we do not propose this medium as one for differential purposes, but rather as one for general cultivation.

Whereas the results reported in the above experiments were obtained with the medium as described, it is possible to vary considerably the proportions of corn and liver, and the method of preparation of the medium, and still to obtain satisfactory results. Corn from 2 to 8 per cent has been used with 1 and 2 per cent liver. Variation within these limits has shown no marked effect upon growth in the medium. However, the low concentrations of corn are not to be recommended, because low viscosity of the mash makes it difficult to get even distribution of the medium in tubing. It also allows escape of the gas and renders less definite the reading of growth of non-starch-fermenting organisms. High viscosity, on the other hand, increases the difficulties of sterilization. It was thought that an extract of liver might be used to replace the dried tissue, but it was found to have no advantage. In other words, it appears that the corn-liver combination is the essential feature of the new medium and that certainly within limits the details of preparation are secondary.

SUMMARY

In corn-liver medium there has been found an inexpensive medium for the cultivation of anaerobic bacteria, which is simple and easy to prepare, which supports the growth of every culture tested so far, which is more efficient in growth initiation from dilute inocula than other media now generally used for anaerobic culture, and which has some differential value within certain groups of anaerobes. It has been used with good results for dilution counts of both vegetative and spore suspensions of anaerobes, and natural mixtures such as soil, lake mud and silage.

The species names used in this paper are those now prevalent in the American literature. The possible identity of cultures of this group bearing different names is not here considered. Such a study is in progress and will be published.

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A MECHANICAL DEVICE WHICH PREPARES AND INOCULATES ROLLED TUBES

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Roll-tubes for the culturing of bacteria were developed by Esmarch in 1886. While the roll-tube method of culturing bacteria is familiar to bacteriologists, it has not found general favour on account of the difficulty encountered in securing a uniform distribution of the culture medium, and on account of the time necessary for their preparation. Esmarch used rolled tubes principally for the procuring of segregated colonies, but also showed that they compared favourably with Petri plates for quantitative analyses.

Wilson (1922) has shown that the roll-tube method possesses certain definite advantages over Petri plates for elaborate counts in statistical work. Gee (1932) has recently used roll-tubes successfully for the critical study of marine organisms where it was necessary to make viable counts at sea; he points out that Petri plate cultures could not be handled conveniently on board ship. It was also with the view of carrying out bacteriological investigations on board a private boat that the author first became interested in roll-tubes. The workers cited have added the inoculum to liquefied culture media, whether gelatin, agar or a mixture of the two, and after mixing have spread the inoculated medium into a film by some method of rotation. Esmarch and Wilson simply rotated their tubes in a horizontal plane, by hand, until the culture medium became solidified. Gee has developed a mechanical spinner which spreads the inoculated culture medium in an even layer over the walls of the tube by centrifugal force. This method has the advantage of turning out uniform tubes free from wrinkles and is more rapid than hand rolling. None of the previous workers has attempted to use roll-tubes for surface inoculation.

The chief advantages of rolled tubes over Petri plates may be summed up as follows: (1) There is considerably less risk of contamination in the case of rolled tubes. (2) Rolled tubes are more convenient to carry. (3) Rolled tubes are much less expensive.

EXPERIMENTAL

When culturing certain types of micro-organisms, it is desirable to inoculate onto the surface of solidified culture media. Petri-plates are suitable when the culturing is carried out in a well-equipped laboratory and when the inoculum can be transported to the laboratory in a fresh condition. For field work however, the Petri-plate method is unsatisfactory and an adequate substitute is desirable. During investigations on undulant fever in Canada, it was found necessary to carry out bacteriological investigations at the place of infection as it was not possible to transport inocula such as blood or milk to the base laboratory in a condition which would insure a satisfactory bacteriological analysis. Poured Petri-plates were found unsatisfactory; first, because of the difficulty in preventing contamination during transportation and, secondly, because the inoculation and distribution of inoculum was a difficult procedure in a private home or poorly-equipped hospital laboratory. Contamination subsequent to inoculation is troublesome, especially when plates must be held for periods of two or three weeks.

In order to overcome such difficulties in this and related work, the author has devised a technique whereby bacteria may be cultured on the surface of culture media inside test tubes. Such a technique necessitated, first, the preparation of films of culture medium on the inside of tubes and, secondly, a satisfactory method of distributing the inoculum over the surface of the solidified medium in the rolled tubes.

TECHNIQUE EMPLOYED

The principle of centrifugal force has been employed to accomplish both the preparation of the culture films and the distribution of the inoculum over the surface of these films. To make this method of distribution possible the agar films were made thickest at the base, gradually becoming thinner towards the surface, thus forming an inverted cone. When the inoculum is placed in the base of this inverted cone and the cone made to revolve at a high speed, the inoculum is forced to climb up the walls. Such a procedure results in the most dense seeding at the base, gradually becoming less dense towards the upper limit. Both the preparation of the rolled tubes and the distribution of the inoculum have been accomplished by the use of the same machine.

EXPERIMENTS WITH VARIOUS ROTATING MECHANISMS

At the outset, 1931, a device similar to that used by Gee (1932) was used for rotating the tubes. While a device which rotates the tubes by connecting a tightly-fitting rubber stopper to a motor was found to produce satisfactory agar films, there were certain serious disadvantages which made the system impractical for surface culture technique. The necessity of pushing down the cotton stopper to a level where the rubber stopper could be tightly fitted was not only inconvenient, but it also necessitated that the agar films be comparatively short. Before inoculation, this cotton stopper must be removed, often a difficult task, and after the addition of the inoculum it must again be inserted to allow the introduction of the rubber stopper for the spreading of the inoculum. Such a procedure is conducive to contamination.

The necessity of having to adjust either the motor or the cooling chamber each time a tube was to be prepared or the inoculum was to be distributed proved a tiring procedure. To overcome the above-mentioned obstacles, a machine embodying a new and very different driving principle was devised.

PRINCIPLE INVOLVED IN DESCRIBED MACHINE

In the present device the tube containing the culture medium in liquid form is supported in an upright position in a body of cooling liquid with the lower end of the tube received in the cavity of a rotary tube-spinning cup. When the cup is rotated at a sufficiently high rate of speed (usually above 1500 r.p.m.)

the centrifugal force developed causes the culture medium to spread over the walls of the spinning tube to form a solidified film gradually diminishing in thickness toward the upper limit. The tube is then removed and stored in a cold place until the inoculum is to be added and distributed over the surface of the prepared film. It is desirable that the spreading of the inoculum be conducive to the growing of well isolated colonies at some location on the prepared film. This is accomplished in the following manner. After addition of the inoculum the prepared tube is dropped into the cooling liquid and engaged with the spinning cup which is made to revolve at a speed of about 2460 r.p.m. Owing to the centrifugal force developed at this speed and to the sloping surface of the film the inoculum is caused to climb up the film to a point near its upper limit. With this procedure the density of the seeding diminishes toward the upper limit of the film and insures the growing of well-isolated colonies.

PRINCIPLE OF AUTOMATIC CLUTCHING[1]

A new principle of hydraulic clutching action resulting from the submergence of the tube-spinning cup in a casing containing the cooling liquid has been employed. Tubes 1" x 8" containing 15 cc. of culture medium, placed in this assembly float in the liquid out of contact with the cup when the latter is at rest or rotating at a slow speed, but are automatically drawn down into the cup by the action of the disturbance set up in the cooling liquid when the cup is rotated at relatively high rates of speed. When the cup is slowed down or brought to rest the action of the liquid is such that the tube is then floated out of the cup to a released position remote therefrom. It will thus be seen that the cup and tube constitute driving and driven members which are clutched together or released by the action of the cooling liquid in response to variations in the operating speed of the cup. In laboratory tests it was shown that at a speed of 1500 r.p.m. there was a decrease in pressure of 2.54 cm. of water at a point

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[1] The author wishes to thank Mr. T. O. Cooper of the School of Engineering, McGill University, for conducting the laboratory tests on the "automatic hydraulic clutch."

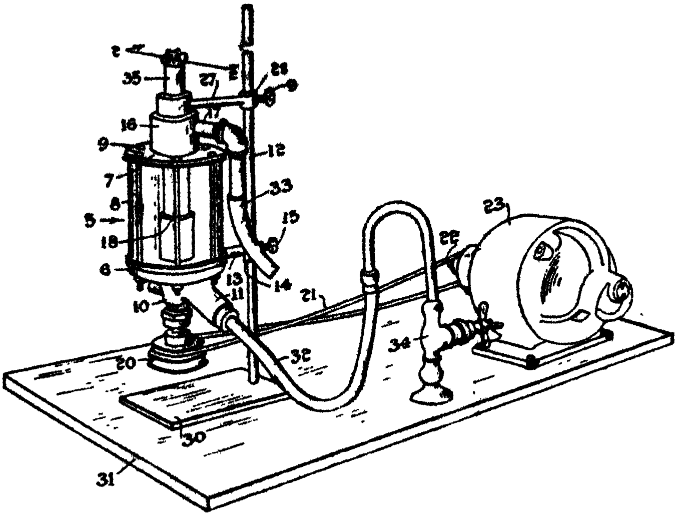

Fig. 1. Perspective View of Apparatus

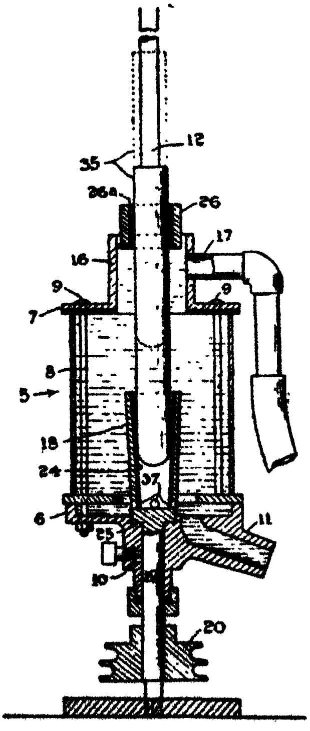

Fig. 2

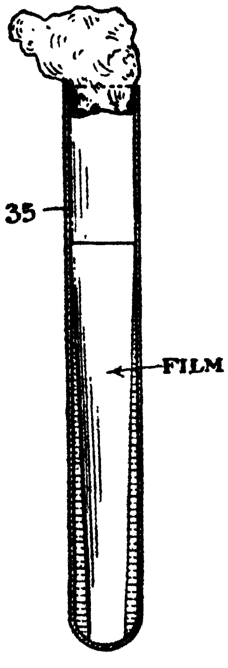

Fig. 3

Fig. 2. View in Vertical Section Taken Substantially Along the Line 2-2 of Figure 1

Fig. 3. View in Vertical Section of Rolled Tube Showing Manner in which Culture Medium is Distributed to Form Film of Varying Thickness

½ inch above the centre of the driving cup. This would be equivalent to the test tube floating 2.54 cm. lower in the fluid, which brings it down into the cup; at higher speeds the decrease in pressure is greater. At speeds above 2000 r.p.m. the machine, when filled with water, will spin empty 1-inch by 8-inch test tubes equally as well as those containing 15 cc. of culture medium.

Figure 1 is a perspective view of the apparatus.

Figure 2 is a view in vertical section taken substantially along the line 2–2 of Fig. 1.

Figure 3 is a view in vertical section of a rolled tube, showing the manner in which the culture medium is distributed to form a film of varying thickness.

Referring to the drawings, *5* designates a casing comprising a cast metal base *6*, a cover *7* and a glass cylinder *8*, the latter being clamped between the base and cover by a series of clamping bolts *9*. The base *6* is provided with a shaft-bearing *10* and a water-inlet passage *11* and is slidably mounted on a supporting standard *12* by means of an arm *13*, sleeve *14* and clamping screw *15*. The cover *7* is provided with a neck extension *16* equipped with an overflow connection *17*.

The casing *5* encloses a driving cup *18* provided with a depending shaft *19* extending downwardly through the bearing *10*. A pulley *20* at the lower end of this shaft is driven, by a belt *21*, from a pulley *22* on the armature shaft of an electric motor *23*.

As shown in figure 2 the cup *18* is slightly conical and is provided with a friction lining *24* of rubber. The bottom of this cup rests in a recess *25* at the upper end of the shaft bearing *10*.

A cylindrical tube-guide *26* is fitted in the neck extension *16* of the cover member in axial alignment with the cup *18*. This tube guide is fitted with a bushing *26a* and is adjustably mounted on the standard *12* by means of the arm *27*, sleeve *28* and clamping screw *29*. The lower end of the standard 12 is screwed into a flat metal base *30* mounted on a main supporting plate *31* which also carries the motor *23*. A constant flow of water through the casing *5* is maintained by means of a water supply pipe *32* and an overflow pipe *33*, the former being connected with the water passage *11* of the base *6* and the latter with the overflow connection *17* of the cover member. The flow through pipe *32* is controlled by a valve *34* mounted on the main supporting plate *31* and adapted for connection with a suitable water supply.

PREPARATION OF ROLLED TUBE

A tube, e.g., *35* with agar in liquid form, is dropped through the tube guide *26* into the body of water contained in casing *5*. Tubes 1-inch by 8-inch containing 15 cc.of melted agar float free from cup *18* when machine is at rest. If now the rotational speed of the cup is accelerated to speeds above 1500 r.p.m. the tube *35* will be automatically lowered into the driving engagement with the friction lining *24*, as illustrated by full lines in figures 1 and 2. If the cooling water is held at temperature of 15°C. and the driving cup is rotated at 1900 r.p.m. satisfactory films are prepared in one and one-half minutes. The shape of the film is clearly shown in figure 3. The tube may easily be removed, while the machine is in motion, by simply grasping the projecting part of the tube between thumb and finger. The prepared tube is placed in a cold room until ready to be used for culturing. It will thus be evident that a number of tubes may be quickly and easily prepared and may be held for considerable periods with little danger of contamination.

ADDITION AND DISTRIBUTION OF INOCULUM

The inoculum may be added to the prepared tube by means of a pipette or hypodermic needle. If desired, inoculum may be added to a whole series of prepared tubes before resorting to the use of the spinning apparatus for distributing the inoculum over the prepared films. In culturing milk and blood satisfactory results have been obtained by rotating the prepared tubes for a few seconds at a speed of 2640 r.p.m. The speed variations in the described set-up has been obtained by placing the driving belt on the correct pulleys. The centrifugal force developed at this speed forces the inoculum, (usually 0.1 cc.) to climb up the sloping exposed surface of the cone-shaped film to a point near the upper limit of the film. As the inoculum is forced upward the film is seeded with the organisms therefrom. This procedure results in the most dense seeding at the base, and a gradual lessening toward the upper limit of the film. Well-isolated colonies should be procured at some location along the agar film. After distribution the tube is removed, as outlined above, and incubated in a vertical position at the temperature desired.

ADVANTAGES OF OUTLINED TECHNIQUE

The described technique may be employed in the culturing of a wide variety of micro-organisms and is especially advantageous for use in field work. The author has found the method well suited for bacteriological investigations on undulant fever. Since Canada is a vast sparsely-peopled country, bacteriological investigations must frequently be carried out at locations hundreds of miles distant from laboratory facilities. Colonies may be picked from these tubes as easily as from Petri-plates. If the tubes are held in a horizontal plane when the cotton stopper is removed, and if careful technique is followed, they may be opened many times without becoming contaminated; a technique difficult with Petri-plates. Inoculated tubes may be held for periods of two to four weeks without danger of extraneous contamination.

Where it is not feasible to employ the machine for the distribution of inoculum, one may simply slowly rotate the prepared tube to which the inoculum has been added, by hand, in an inclined position, thus causing the inoculum to flow over the surface of the culture medium. Such a technique, however, results in less satisfactory results. Where electric current is not available the author has employed an ordinary 6-volt battery and an automobile starting-motor as a power source.

Experiments are now being conducted to demonstrate the relative efficiency of the roll-tube and Petri-plate methods in the qualitative and quantitative bacteriological analysis of various liquids. Investigations conducted to-date show that these methods give closely similar results when used for the quantitative determination of *Br. abortus* in milk.

SUMMARY

A mechanical device which makes feasible the employment of rolled tubes, instead of Petri-plates, for culturing bacteria on the surface of solidified culture media has been described. The action of a new type of automatic clutch, which is incorporated in the mechanical device, is described.

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THE EFFECT OF DEUTERIUM ON THE GROWTH OF YEAST

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Water composed chiefly of deuterium, the heavier isotope of hydrogen, has been found unsuitable for living organisms when they are placed in it without gradual adaptation. As with most poisonous substances, less injury, some stimulation, and finally no effect should be obtained with increasing dilution of the heavy water. Barnes (1933) gives references to earlier investigations on this subject.

A pure strain of yeast, *Saccharomyces cerevisiae* Hansen, was grown in Williams' medium[1] made with deuterium containing water[2] having a specific gravity of 1.000061 (1 part in 2000) and in the same medium made with distilled water using the controlled methods described elsewhere (Richards, 1932a; Richards and Jahn, 1933).

No significant difference was found in the number of cells per unit volume during the growth in the two waters as the differences noted were only about one-fourth the standard error of these differences. Nor were significant differences found in the percentage of buds and of injured cells, or in the mean cell size of the two populations of yeast grown under these conditions. The cells grown in the heavier water were slightly more uniform in size than those grown in distilled water as the coefficient of variation of the former was about 8 per cent less than that of the latter.

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[1] Williams' medium contains sugar 20 grams; $(NH_4)_2SO_4$, 3 grams; KH_2PO_4, 2 grams; Asparagin, 1.5 grams; $CaCl_2$, 0.25 gram; $MgSO_4$, 0.25 gram; and distilled water 1000 ml.

[2] The heavy water was furnished to me through the kindness of Dr. T. C. Barnes and for his description of it *cf. Jour. Am. Chem. Soc.*, 1933, 55, 4332. The specific gravity was measured to $\pm 5 \times 10^{-6}$. This is a greater uncertainty than advised by Washburn, but the difference found does indicate a significant concentration of heavy water.

Measuring the growth of the populations with a photoelectric nephelometer gives a criterion of growth depending on the number of cells present, the distribution of the sizes of the cells and the optical density of the cells. Table 1 shows an increased growth of 11 per cent (standard error 1.5 per cent) for all of the determinations of three series of the yeast grown in the heavier water above that of the yeast grown in distilled water. With no significant differences in the number of cells in the two populations this indicates increased total volume of the cells with or without cytologically more dense cells. The total volume of the cells from the population grown in the heavy water is greater than that from the control population in distilled water (table 1), when they are centrifuged into calibrated volume tubes. This difference increases with the age of the populations. As the mean cell size is not very different, less close packing occurred in centrifuging the yeast obtained in the heavy water. The rela-

TABLE 1

Growth of yeast in heavy water in per cent of the growth of the control yeast in distilled water

SERIES	NEPHELOMETER		AGE	VOLUME	DRY WEIGHT
	N*	Per cent			
			hours	*per cent*	*per cent*
130	24	108			
139A'†	16	103	106	114	111
134	30	117	114	117	120
134		Av. 111*	143	159	135
139B'†			106		86
139C'†			106		92

* Average weighted in terms of the number of measurements (N).

† Series 130, 134 and 139A' in heavy water of specific gravity 1.000061; Series 139B' one-fourth heavy water and three-fourths distilled water; Series 139C' one-half of the above heavy water and one-half distilled water.

tion between the several criteria of yeast growth will be given elsewhere.[3]

The dry weight of the yeast is greater when the cells are grown in water containing the heavier isotope of hydrogen and this difference also increases with the age of the populations (table 1). The ash content of the yeast grown in this dilute heavy water is less (9.5 per cent) than that of the control yeast (11.3 per cent) showing that the difference in the dry weight is due to non-mineral storage material. The glycogen content of the yeast from the heavy water cultures was nearly twice that of the controls but their nitrogen content was only 69 per cent of that of the control yeast populations. The increase of glycogen and loss of nitrogen is characteristic of old populations of yeast and is also observed in the ageing or ripening of commercial yeast.

The greatest effect on the yeast is shown by the nephelometric method to occur at the two times when the rate of population growth is greatest for the two cycles of growth which occur when this yeast is grown in the Williams' medium at 28°C. At these two times the cells are smaller and have a proportionately greater surface for the exchange of nutrient material with respect to their volume (fig. 1, *A*, *B*). The effect at this time leads to an earlier reorganization within the cells making those grown in the heavy water appear to be aged with respect to the controls. This ageing, or cellular reorganization, normally occurs as the culture medium becomes less favorable, from the exhaustion of food and the accumulation of the toxic excretion products of the cells. Hence, the effect of the water containing deuterium is unfavorable from the standpoint of a maintained constant environment. On the other hand, if the criterion of growth is bulk or weight, the dilute heavy water may be said to stimulate the yeast growth.

A single experiment made with two dilutions of the heavy water indicated that the optimum effect occurred with about half of the concentration of the heavy water used in my experiments (i.e., one part in four thousand). Further dilution gave less difference as shown by the nephelometric criterion plotted on figure 1, *C*.

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[3] An abstract appears in *Anat. Rec.*, 1933, 57 (4) Suppl. 36 and the details will be published in the near future.

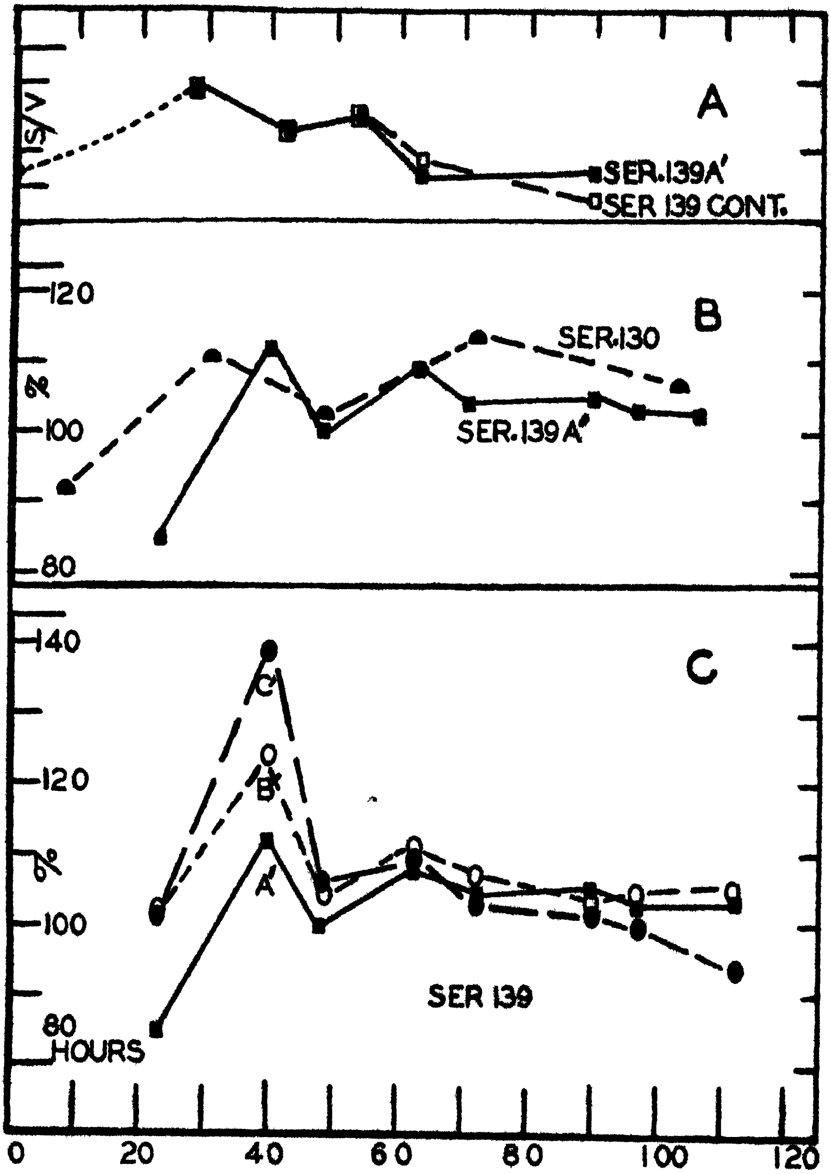

FIG. 1. *A*, the relation of the surface to the volume of the average yeast cell during growth. (Ser. 139 *A'* in heavy water, 139 cont. the control in distilled water). *B*, the growth of yeast in heavy water as a percentage of the growth of the control cultures in distilled water for two independent experiments. *C*, the growth in heavy water, *A'*; in 1 part heavy water to three parts distilled water, *B'*; and in 1 part heavy water to 1 part distilled water, *C'*.

Pascu (1934) has found that the fermentation of glucose by yeast is much less in concentrated heavy water (100 per cent and 60 per cent deuterium oxide), but Macht and Davis (1934) report no difference with a concentration of heavy water about the same as used in my experiments. Barnes and Larson (1933) have reported a 10 per cent decreased liberation of carbon dioxide by zymin preparations from sugar in solution in the same strength heavy water as that used here.

A greater effect is observed on the first cycle of growth when the energy for growth and maintenance appears to come mainly from the fermentation of the sugar in the medium. The effect during the second growth cycle is less and the energy then comes mainly from an oxidative process. The effects occur when the ratio of surface to the volume of the cells is greatest. Thus, the presence of the heavy isotope of hydrogen may alter the permeability of the cell or may have more effect on the enzymes of the cell. The result is increased bulk of yeast and earlier ageing of the cells. While a change in the bound water of the cell colloids is not excluded it seems to be a less satisfactory explanation than an effect on the cell enzymes (Barnes and Larson, 1933). The results of the experiments are marked, but the stimulation observed is much less than that known to occur with yeast growth stimulants such as thallium (Richards, 1932b), or some of the "bios" preparations (Williams and Truesdale, 1931).

Until more heavy water becomes available it will not be possible to determine the optimum concentration, the effects on fermentation and respiration, and the extent to which the yeast may be adapted to greater concentrations of the heavy isotope of hydrogen. The amount of protium which may be replaced by deuterium in the cell must be determined under carefully controlled conditions. Until this information becomes available from critical, quantitative study it is premature to speculate further on the nature of the effects observed in these experiments or to generalize on the rôle which the newly discovered isotope of hydrogen may play in living matter.

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AN INVESTIGATION OF THE BACILLUS PASTEURI GROUP

I. DESCRIPTION OF STRAINS ISOLATED FROM SOILS AND MANURES

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In 1889, Miquel briefly described *Urobacillus Pasteurii*, a sporing bacillus found in rivers and sewage which brought about an exceptionally active decomposition of urea. Beijerinck (1901) gave a fuller account of an organism isolated from soils and dust which he considered to be *Urobac. Pasteurii*; but the first detailed description of the bacillus was published by Löhnis and Kuntze (1908) who referred to the organism as *Bacillus Pasteuri* (Miquel) Migula. A number of other urea-decomposing bacteria which have been described are difficult to distinguish from *B. Pasteuri* and undoubtedly belong to the same group. In this category may be mentioned *Urobac. Duclauxii* and *Urobac. Maddoxii* (see Miquel, 1904), *Urobac. Freudenreichii* Miquel which was fully described by Löhnis (1905) as *Bacillus Freudenreichii* (Miquel) Migula, *Urobac. leubei* Beijerinck, *Bacillus probatus*, A. Meyer et Viehoever, *Urobac. psychrocartericus* Rubentschik and *Urobac. hesmogenes* Rubentschik. The classification of these bacteria is still one of the least satisfactory parts of systematic bacteriology. The uncertainties in classification appear to have arisen partly because certain species were imperfectly described and partly because most workers who made detailed descriptions of their organisms worked with comparatively few strains. It is noteworthy that Geilinger (1917), who investigated a larger number of strains than any of his predecessors, experienced more difficulty than others in classifying the organisms. Since the work of Beijerinck (1901) and Löhnis and Kuntze (1908), the most clearly characterised organism of the group has generally been considered to be *B. Pasteuri*; but the distinctions between this type and other sporulating urea decomposers have never been precisely defined.

A consideration of the literature dealing with this group of organisms suggested that their classification could only be clarified by a comparative study of a large number of strains supplemented by an investigation of their variability under the conditions of artificial culture. Work was undertaken on these lines and the results of a study of 94 freshly-isolated strains are presented in this paper. The variability of the pure cultures was explored partly by the separation of variants by plating and partly by the reëxamination of stock cultures which were transferred every two months. Fifty-three strains were cultivated on urea agar for four years: a proportion of them were examined at intervals and all were investigated again at the end of the period.

SPECIAL MEDIA AND METHODS

The media found to be most suitable for studying pure cultures are bouillon, agar and gelatin in which 2 per cent urea is dissolved immediately before sterilisation. They will be referred to as urea bouillon, urea agar, etc. These media contain 1 per cent peptone and 1 per cent meat extract. Lower concentrations are less satisfactory, probably on account of smaller buffering capacity in the neighbourhood of pH 9. In order to obtain media free from precipitates produced by the breakdown of urea during sterilisation, the bouillon is prepared by adjusting the reaction to approximately pH 8.5 by adding NaOH and autoclaving: after filtration the reaction is brought back to approximately pH 7 by adding HCl and the remaining ingredients are dissolved. Intermittent steaming is the most suitable method of sterilisation. Urea readily decomposes in the autoclave and although this may not destroy the growth-promoting properties of the media, it leads to excessive precipitation. Media containing urea sterilised by filtration do not encourage growth to the same degree as those in which the urea is sterilised by heat. A freshly prepared medium is superior to one which has been stored for some time. Agar containing 0.25 to 1.0 per cent urea is satisfactory for maintaining stock cultures, especially those of the less active strains, but is inferior to 2 per cent urea agar as a plating medium.

With the object of securing a representative series of organisms, pure cultures were isolated from a variety of sources by different methods. In every case primary cultures were prepared in media containing 5 or 10 per cent urea in order to utilise the special tolerance of ureaclastic organisms to high concentrations of this compound and to the ammonium carbonate which they produce from it. Three main methods were used.

1. Enrichment cultures were prepared by inoculating urea-containing solutions and incubating at 22°, 30° or 37°C. The media used were bouillon and soil extract (1 kgm. garden soil and 1 l. tap water autoclaved to 22 pounds, filtered, the filtrate made up to 1 l. and 0.05 per cent K_2HPO_4 added), the latter with or without the addition of 1 per cent glucose or glycerol. Usually a 5 gram quantity of soil or manure was added to 50 ml. of medium in an Erlenmeyer flask. In a few cases deep tubes were used with the object of restricting aeration. The course of the decomposition was followed by periodically titrating 1 ml. quantities of the cultures against standard H_2SO_4 and isolations were made at different stages. All the different forms of enrichment culture just described appear to lead to the same result: they allow the active urea-decomposing bacteria in the sample to grow more rapidly and outnumber inactive organisms. Even when isolations were made early in the development of the cultures, the majority of the organisms secured proved to be active types. Variations in the medium, in the temperature of incubation or in aeration did not appear to influence the types of bacteria which developed except that it was necessary to add about 10 per cent urea to bouillon and about 5 per cent urea to soil extract in order to prevent completely, during the early stages of incubation, the growth of organisms which do not decompose urea.

2. In order to isolate organisms which might be incapable of multiplying rapidly in enrichment cultures and yet occur in large numbers in soils, the dilution method was used. Dilutions of soils were prepared and tubes of 10 per cent urea bouillon or

5 per cent urea soil extract were inoculated from a series of dilutions. In view of Millard's (1912) observations on the use of the dilution method for soil bacteria, about 0.5 gram of soil was sterilised in each tube by autoclaving prior to adding the media and sterilising by intermittent steaming. The inclusion of soil in the media had, apart from its possible nutritive properties, the advantage that if any appreciable decomposition of urea took place the culture became brown as a result of the solution of humus by ammonia. The organisms isolated from the highest dilutions containing urea decomposers were invariably among the least active types.

3. Direct plating of soil or soil dilutions on agar was used in a search for organisms for which methods 1 and 2 are unsuited. Flasks containing 100 ml. of nutrient agar (2 per cent agar, 1 per cent peptone and 1 per cent meat extract) were prepared and immediately before use the medium was melted, 5 or 10 per cent crystalline urea was added, and, after steaming for 10 minutes, the medium was cooled and used for plating. The soil or soil dilution was either mixed with the melted agar or spread on the surface of the medium after hardening. Colonies of urea-decomposing bacteria generally produced precipitates of microscopic crystals in the agar surrounding them as a result of the intense alkalinity developed; but colonies which appeared late or tended to spread did not always show this appearance. The agar containing 10 per cent urea suppresses practically all organisms which do not decompose urea but it is also more inhibitive to ureaclastic bacteria than the less selective 5 per cent urea agar. The cultures isolated by direct plating of soils were invariably types possessing feeble urease activity.

Most of the strains were isolated on 2 per cent urea agar. Beijerinck's (1901) urea yeast-extract gelatin, which is also a suitable medium, was used for a few isolations. The gelatin possesses some advantage in showing crystal formation around the colonies of urea-decomposing organisms. Several other solid media were tried but were found to be unsatisfactory. Colonies appearing on primary plates were replated at least once before cultures were assumed to be pure. The urease activity of pure cultures was tested in bouillon containing 2 per cent urea, the course of the decomposition being followed by titrating 1 ml. quantities of the cultures against standard acid. Tests for residual urea were made, when necessary, with soybean urease.

The majority of the bacteria which were isolated belong to the *Bacillus Pasteuri* group and they alone will be described in this paper. Isolations were made from 38 samples taken from 6 types of soil, 32 strains being obtained from enrichment cultures, 19 from liquid media inoculated with high dilutions and 37 by direct plating. The remaining 6 strains were isolated from enrichment cultures inoculated with farmyard manure.

DESCRIPTION OF PURE CULTURES

It may be indicated here that the possibility of classifying this group of bacteria in clearly defined species is not promising and the only form of description which appears to be satisfactory is one which surveys the group as a whole. Accordingly, the series of recently-isolated strains will be described as a single entity, but reference will be made to any observations which might be of importance for purposes of classification.

Microscopic characteristics

Morphology. The organisms are rod-shaped with rounded or slightly pointed ends. Chain formation may be observed in preparations of living cells, but seldom in stained films. There are two main morphological types. In one (plate 1, figs. 1 and 2) the stained cells are 0.5 to 1μ thick and variable in length: spherical or slightly ovoid spores, which are usually greater in diameter than the sporangia, are formed chiefly in a terminal or sub-terminal position. In two strains (fig. 3) the spores are exceptionally large and measure 1.5μ or more in diameter. In the second main type (figs. 4 and 5) the cells are comparatively short and frequently almost coccoid: they are seldom more than 3μ in length and they vary from 0.6 to 1.5μ in thickness. In these strains the spores are ovoid to elliptical: they are formed in a central or excentric position and they may occupy most of the cell interior.

The shape and size of the vegetative cells are moderately constant strain characteristics so long as stock cultures are maintained in the usual way, but stable modifications in morphology may be produced when variants are isolated by plating. Organisms which grow almost entirely as long undivided filaments (fig. 6) may be obtained in this way. The shape of spore appears to be a stable character but its value as a basis of classification is reduced by two circumstances. Firstly, there is no definite division between the two main types described above for every possible intermediate form has been encountered and, secondly, among organisms of this group the capacity to form spores usually becomes much reduced and may be completely lost during artificial cultivation. Spore formation occurs most readily on solid media comparatively poor in nutrients and approaching in their reaction the limiting acidity tolerated by the organisms. Media suitable for eliciting the production of spores are potato and ordinary agar prepared from a dilute bouillon, provided growth can be induced on these substrates. The heat resistance of the spores is variable. A few cultures when tested immediately after isolation contained spores which survived exposure at 98° to 100°C. for fifteen minutes. Others were destroyed at the same temperature almost immediately. In general, the more elongate the spores, the greater is their resistance to heat.

Cells of irregular shape are seldom formed. A few strains occasionally produce spherical to spindle-shaped forms 2 to 5μ in diameter. These are probably the cells which Miquel (1905) considered to be typical of *Urobac. Maddoxii.* Certain strains or strain variants—chiefly those which grow as long rods—exhibit a tendency to form small non-motile coccoid bodies (plate 2, fig. 7). These bodies develop from minute buds and correspond in their characteristics to one of the types of bacterial gonidia. Strains which no longer form heat-resistant spores may produce spherical bodies (fig. 8) which are usually smaller than spores and are unstainable in their mature condition except by spore-staining methods. These structures are destroyed immediately at 80°C. They generally originate as Gram-positive buds and they correspond therefore to "regenerative bodies" as defined by Löhnis (1921). There can be little doubt, however, that in this group of bacteria they represent a degraded form of spore.

Motility. With one exception—an organism which has not been observed to form spores—all the strains were motile when examined soon after isolation. The arrangement of the flagella is peritrichous, but in some strains few of the cells appear to be furnished with more than 2 flagella. Several of the variants isolated from particular strains by plating proved to be non-motile. These were chiefly long slender rods and the filamentous types shown in figure 6, but two of them were comparatively short rods.

Staining. The rods stain readily with aqueous fuchsin and seldom show any differentiation of their protoplasm. The reaction to Gram's method of staining is variable. The thicker rods are generally Gram-positive but the thinner organisms are easily decolorised and may be frankly Gram-negative. With modifications of Gram's method in which acetone is used most strains are decolorised instantly.

Conditions of growth

The optimum temperature varies among different strains from about 25°C. to about 37°C. Strains showing the highest optimum have a maximum temperature of about 42°C. Those with the lowest optimum do not grow at 30°C. or at higher temperatures. The minimum temperature of the latter types is about 0°C. Among the strains which have temperature maxima under 30°C. there are no examples of organisms forming elongate spores or capable of producing a rapid decomposition of urea.

The organisms are aerobic but the oxygen requirement, as shown by stab and shake cultures in agar, varies to some extent among different strains.

The results of experimental work on nutrient requirements and on the effect of reaction will be presented in a subsequent paper.

Most strains, including the non-sporing types, remain alive on urea agar for several months. The viability, especially of types which produce a thin growth, is considerably prolonged if the agar contains a low concentration of urea (0.25 to 0.5 per cent) and if drying is prevented. If growth is secured on ordinary agar, the organisms remain alive for several years.

Cultural characteristics

Urea-containing media are generally referred to in the following account of cultural characters. Strains which decompose urea actively are almost incapable of developing in ordinary neutral media, while only a few of the least active strains grow readily in these substrates. In the case of strains capable of growing in ordinary media, the cultural characteristics are not appreciably modified by the presence of urea.

Colonies on urea agar. The following are the chief types of surface colony produced by different strains or by variants of the same strain:

Type 1. Small (up to 1 mm.), round, translucent; bluish by transmitted light. Microscopically finely granular; edge entire. This type is illustrated on plate 2, figure 9.

Type 2. Larger (up to 3 to 4 mm.), round, grey and glistening; becoming thick, opaque, and by transmitted light yellowish-grey. Microscopically finely granular without other features, later opaque; edge entire. Illustrated in figure 10.

Type 3. At first translucent and granular by transmitted light; later opaque and yellowish in the centre, and translucent and granular in the marginal zone; edge ragged; up to 1 cm. in diameter. Microscopic structure resembles folded hair. Illustrated in figure 11.

Type 4. A series of thin colonies varying from the type illustrated in figure 12, which has a comparatively smooth surface, to much thinner and more irregular forms which show a folded hair structure microscopically.

The different types of colony appear to be determined mainly by the morphology of the organisms. Colonies of type 1 are generally produced by the smaller rods, those of type 2 by short thick bacilli, while those of types 3 and 4 are formed by long rods and filaments. The form of colony, within the range of types described, can be of only limited value from the systematic standpoint. It is entirely unrelated to the biochemical properties of strains, it exhibits every grade in type between the extremes, and it is not specially stable. After the isolation of a pure culture, the form of colony tends to change in the direction of the "rough" types 3 and 4, a process which may be greatly accelerated by making serial transfers in urea bouillon and isolating distinctive variants.

Colonies on urea gelatin. Colonies formed on gelatin by different strains form a series of types similar to those on agar. The distinctions between types are generally more clearly marked in gelatin colonies, but a classification of the various forms is complicated by the variable rate of liquefaction of the medium and the modifications of colony structure thus produced. The surface colonies may either develop into circular liquefactions or form hollows or they may show no evidence of acting on the gelatin. Microscopically, they may become ciliated, hairy, or diffuse, or they may produce flame-like outgrowths, depending on the rate of liquefaction. Deep colonies are usually dense and round, occasionally irregular. At a later stage they may produce microscopic hair-like or twisted outgrowths or lobes. The colonies of some strains may resemble those of Rubentschik's (1926) *Urobac. hesmogenes.*

Urea agar slope. Conforming to the type of colony, the growth on slopes varies in different strains from a thin transparent layer to one which becomes thick and yellowish-grey by transmitted light. The growth has a glistening surface and may be smooth or may have a granular appearance. There is no tendency to rapid spreading.

Urea gelatin stab. The stab growth is at first thread-like, but most strains later form outgrowths from the upper part. The production of outgrowths is not a constant characteristic of particular strains for it depends in part on the composition of the medium. The surface growth is similar to colonies in Petri dishes. The rate of gelatin liquefaction varies greatly among different strains but is never rapid. The most active strains produce in seven days a small saucer-shaped liquefaction which later becomes cup-shaped and then cylindrical. Many strains form an empty cup-shaped or napiform hollow, from which the liquid evaporates almost as rapidly as it is formed, unless the tubes are closed. The least active strains may produce only slight softening of the surface after several months in capped tubes and may show no action on some samples of the medium within one year. Two strains have failed entirely to produce any action on gelatin. The rate of liquefying gelatin does not correlate with any other characteristic of the organisms.

Urea bouillon. Most strains produce a turbidity of varying intensity and later a slimy sediment. A few form a soft pellicle which readily breaks up and sinks in flocculent masses.

Potato. Usually no growth appears. Strains which grow fairly readily on ordinary agar may produce some grey to brown growth if the medium is heavily inoculated and especially if much ammonia is being formed in the incubator.

Biochemical characteristics

Milk. Usually no change takes place. The strains which grow most readily on ordinary media may, if heavily inoculated into milk, slowly produce an alkaline reaction. This change may be followed by a slight discoloration after six weeks or more at the optimum temperature of the organism, and a slow peptonisation may occur during subsequent months. Most strains grow fairly readily in milk to which 2 per cent urea has been added after sterilisation. In this medium the preliminary clearing, brought about by the formation of ammonia, is followed by a slow digestion of proteins and the milk is converted into a semi-clear, brownish liquid after several weeks. The reduction of litmus in milk is slow and variable.

Nitrates. In urea bouillon or semi-solid agar some strains reduce nitrates to nitrites and others do not. Nitrites are not reduced. Nitrate-reducing ability is not associated with any other character which has been found to distinguish different strains.

Indol is not formed as a rule but certain strains produce traces in tryptophane-containing media.

Diastatic action and the formation of *hydrogen sulphide* are not demonstrable by the usual methods.

Glucose bouillon is rendered alkaline by most strains capable of growing in the medium. Parallel cultures in ordinary bouillon generally become alkaline more rapidly, thus indicating that some acid is formed from the sugar. A few strains produce a slight acidity (pH 6.5 to 7).

Urea-decomposing activity. There is a wide variation in the rate of action on urea. The most active strains completely decompose 2 per cent urea in bouillon within twenty-four hours at their optimum temperature, even when the medium is only lightly inoculated. The least active strains are incapable of ammonifying the same concentration of urea within ten days; and between the two extremes there are strains which exhibit all gradations of urease activity. There is no connection between rapidity of growth in the medium and urea-decomposing ability. Both vigorously- and weakly-growing strains may produce either an active or a slow decomposition. This observation suggests that Viehoever's (1913) tedious method of characterising these organisms by finding the relation of multiplication to urea hydrolysis is unsatisfactory. Several observers have reported on the loss of urease activity in different bacteria, but in the Pasteuri group the ureaclastic property appears to be a relatively stable characteristic. Of 53 strains which were cultivated on agar for four years, only one—an organism which at the end of the period was no longer motile and tended to grow in long filaments—lost the ability to produce a titratable alkalinity in urea bouillon. With this exception, the greatest reduction in activity observed was a lengthening of the time required for the complete decomposition of 2 per cent urea in bouillon, from under twenty-four hours to nearly four days. Some irregularity occurs in these tests for the results are influenced by the length of the lag period, which chiefly depends on the amount of inoculum and on the freshness of the medium.

Ureaclastic activity appears to be one of the few possible characters upon which to base a classification of the group. It possesses the disadvantage of arranging strains in an unbroken series, but it is correlated with media requirements and, to some extent, with morphology. The greater the urease activity of a strain, the more unsuitable are the ordinary bacteriological media for its cultivation; and none of the strains which form elliptical spores bring about a rapid decomposition of urea.

CLASSIFICATION OF RECENTLY-ISOLATED STRAINS

Careful consideration has been given to the problem of classifying the series of strains described here and of identifying the different forms with previously-described types. A number of the organisms appear to correspond completely with *B. Pasteuri* as described by Löhnis and Kuntze (1908) and others with *B. Freudenreichii* as characterised by Löhnis (1905). *Urobac leubei* Beijerinck (1901) and certain of Rubentschik's (1926) organisms also appear to be represented. It is impossible, however, to allot every strain to a named species. The earlier investigators, working at different dates, did not employ the same criteria for characterising their species, and in certain cases it is impossible to decide whether they were describing the same or different types. In addition, the series of strains examined in this work appears to embrace a wider range of types than that represented by published descriptions. It is obvious that the classification of the group requires revision.

The observations cited in the description indicate that the recently-isolated strains cannot be grouped easily in well-defined types. Clear divisions are lacking between the extreme morphological and cultural forms, while biochemical characters are associated in almost every possible combination. With the object of providing information which would be useful in classifying the organisms the variability of the strains under laboratory conditions was investigated. The chief changes observed in stock cultures maintained on agar for four years were a decrease in spore-forming ability, some reduction in urease activity, and a change, in the case of certain strains, in the form of colony to a "rougher" type. A few representative strains were employed in attempts to stimulate variation by making serial transfers in urea bouillon and plating the cultures on urea agar. Each of the strains submitted to this procedure produced more than one type of colony, there being a general tendency to the production of "rougher" colonies. Cultures derived from distinctive colonies were subjected to further study, and a proportion of them appeared to be stable variants. This work showed that the differences between strains in respect of the morphology of vegetative cells and the characters of growths on solid media are not constant and are therefore unreliable as an aid to classification. One strain produced the entire series of colony types illustrated on plate 2 and also intermediate forms. The shape of spore was never observed to change, but most stabilised variants lost the ability to form spores. The physiological properties appeared to be the most constant characters of the organisms. Where differences in physiology among the variants of a strain were recognised, they were chiefly of a quantitative nature. The experiments therefore shed no light on the irregular distribution of biochemical characteristics among different strains.

It is impossible to avoid the conclusion that a natural subdivision of this group of bacteria cannot be made and any classification must be arbitrary. Nevertheless, it seems to be desirable to suggest some form of subdivision which will permit of reference in a general way to the salient types within the group. The most suitable proposal appears to be the separation of organisms which decompose urea actively, and are incapable of growing in ordinary neutral media, from the remaining types. The former produce spherical or slightly ovoid spores. The latter might be divided into those which form approximately spherical spores and those which produce ovoid to elliptical spores. The problem of naming these types cannot be dealt with here, but a future paper of this series will contain a report on a number of cultures which were preserved by previous investigators, and the evidence presented will be used in making proposals on nomenclature.

SUMMARY

A description is given of 94 strains of urea-decomposing bacteria which may be placed in the *Bacillus Pasteuri* group. The organisms were isolated from a variety of soils and from manures (1) by means of enrichment cultures in media containing 5 or 10

per cent urea, (2) by inoculating urea-containing liquid media with high dilutions of soils, and (3) by direct plating of soils on agar containing 5 or 10 per cent urea. Method (1) permits of the isolation of the most active urea-decomposing bacteria in a sample, while methods (2) and (3) yield cultures of the most numerous and generally the least active organisms. The group consist of a series of strains among which there appear to be no natural divisions. Strains which decompose urea actively are almost incapable of developing in ordinary neutral media but a few of the least active organisms grow readily in these substrates. Individual strains exhibit variations in the morphology of vegetative cells and spores, the optimum temperature (from about 25° to about 37°C.), the form of colony, and the action on gelatin and nitrates. The variability of pure cultures was explored partly by the separation of variants by plating and partly by re-examining stock cultures at intervals. The study of colony variants proved that differences between strains in respect of cell morphology and the characteristics of growths on solid media are not constant, but the physiological properties of variants are in general identical with those of their parent strain.

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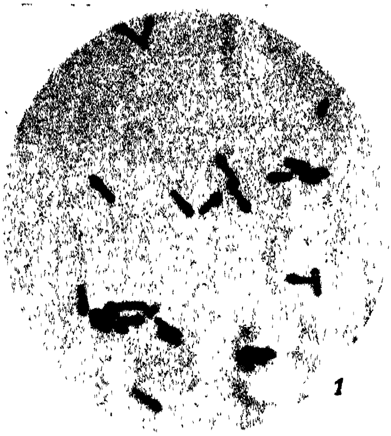
PLATE 1

Preparations from cultures on agar containing urea or ammonia, incubated at 30°C. for one to three days. Stained with cold aqueous fuchsin. Magnification ×1000.



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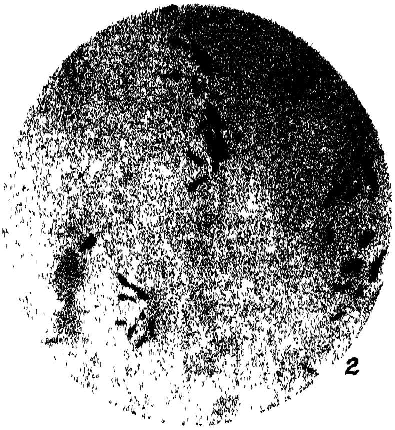

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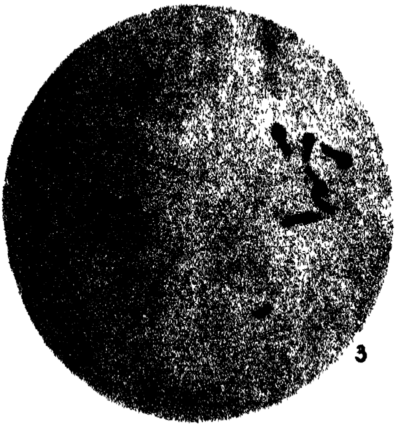

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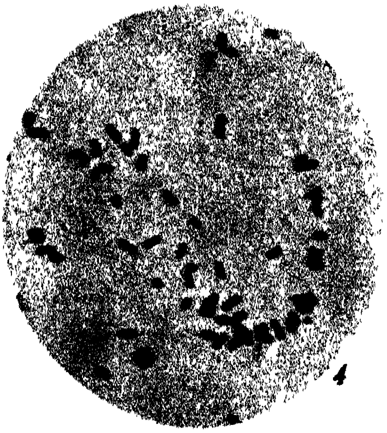

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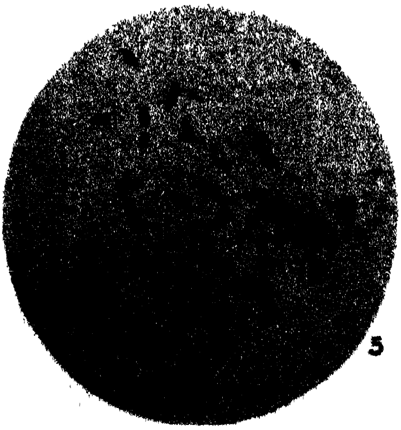

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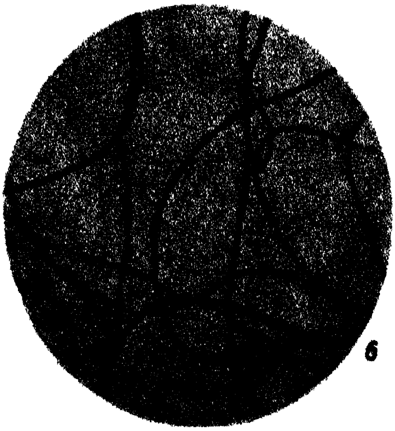

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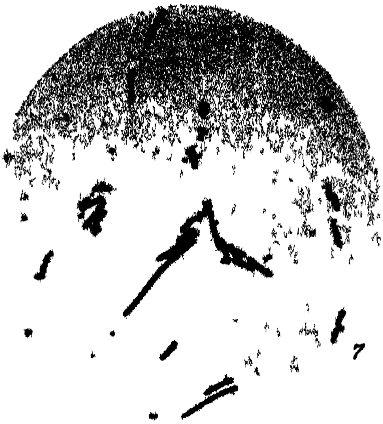
(Gibson. Investigation of Bacillus Pasteuri group)

PLATE 2

Figs. 7 and 8. Preparations from cultures on urea agar incubated at 30 C. for two days (fig. 7) and three days (fig. 8). Stained with cold aqueous fuchsin. Magnification ×1000.

Figs. 9 to 12. Colonies on urea agar incubated for three days at 30 C. Magnification ×10.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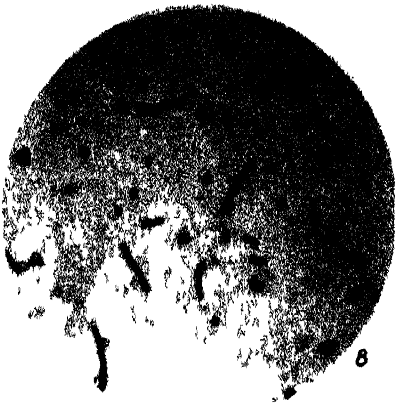
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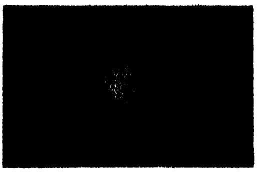
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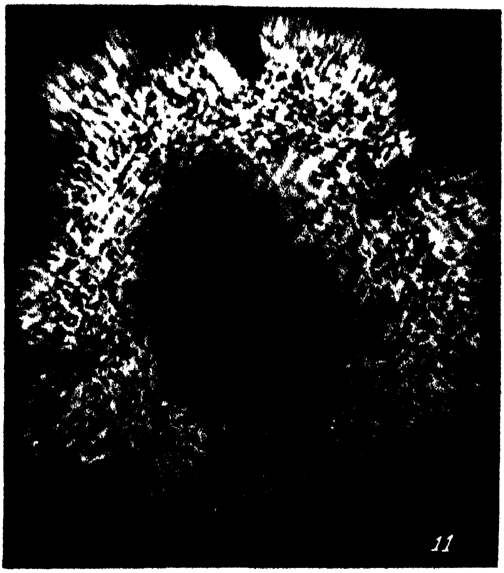
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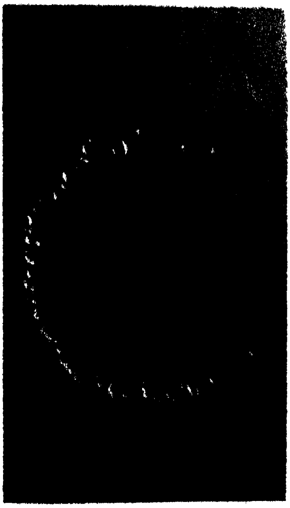
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(Gibson Investigation of Bacillus Pasteuri group)

AN INVESTIGATION OF THE BACILLUS PASTEURI GROUP

II. SPECIAL PHYSIOLOGY OF THE ORGANISMS

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In the first accounts of *Urobacillus Pasteurii* by Miquel (1889, 1898) the organism was described as having been cultivated in bouillon and peptone media containing various concentrations of urea. Beijerinck (1901) found that the bacillus would not grow on the ordinary laboratory media, but developed in bouillon containing 10 per cent urea, or an agar and gelatin to which 2 per cent urea and 0.3 per cent ammonium carbonate were added. He was unable to obtain growth of the organism in urea peptone solutions unless Chapoteau peptone, which was used by Miquel, was employed. Simple media containing, in addition to urea, asparagine and glucose, or ammonium salts of organic acids, did not support growth. Löhnis and Kuntze (1908) used bouillon, agar, and gelatin, each containing 2 per cent urea, for the cultivation of the organism, and confirmed Beijerinck's observations concerning the unsuitability of media which contained no urea or protein. It became generally accepted that the most important criterion for the recognition of *B. Pasteuri* was its inability to grow on ordinary media without added urea. Later work by Viehoever (1913), however, cast doubt on the necessity for urea, and this author observed growth of the organism in much simpler media than those used by Beijerinck and Löhnis and Kuntze. Viehoever described good growth in an ammonium carbonate mineral solution containing glucose and asparagine as the only organic compounds. In the same medium without glucose the organism was still capable of growing, but less vigorously. Viehoever added either urea, ammonium carbonate, or sodium carbonate to all the media he used.

There appears, therefore, to be considerable uncertainty concerning the environmental and nutritive requirements of *B. Pasteuri*, especially with regard to the necessity for urea. The function of urea in the metabolism of the organism has not been examined experimentally. It has, however, been established that the bacillus requires alkaline media, and a consideration of the literature suggests the possibility that urea merely serves to produce a strongly alkaline reaction through its breakdown to ammonium carbonate. This change would occur to some extent during the heat sterilisation of artificial media, and would later be completed by the urease activity of the cell.

THE EFFECTS OF UREA AND AMMONIA ON GROWTH

In this investigation it was observed that most of the strains usually failed to develop from light inoculations in media prepared from one per cent peptone and one per cent meat extract, and adjusted to pH 7.0 to 7.5. If the media were heavily inoculated, many strains initiated growth after incubation for several days, or several weeks, and having started, the organisms multiplied rapidly and the quantity of macroscopic growth frequently exceeded that formed on the same medium to which urea had been added. Evidently the heavy seeding enabled the cells to produce conditions suitable for their multiplication. When the organisms succeeded in growing in ordinary bouillon or agar, the cultures became distinctly alkaline, and the possibility that these substrates might be suitable for growth if made sufficiently alkaline was investigated. Tests were made with bouillon varying in alkalinity up to pH 8.5, but many strains failed to initiate growth at any reaction. Tests were then carried out with samples of bouillon of different reactions, to which 2 per cent urea was added. For this purpose, the urea in concentrated aqueous solution was sterilised by filtration and added aseptically to the tubes of bouillon, thus avoiding complications arising from the decomposition of urea by heat. Small and fairly uniform inoculations were made by thoroughly emulsifying growth from agar in M/15 phosphate buffer of pH 8, so that a definite but faint turbidity was produced, and transferring a loopful to each tube of bouillon. The results were unexpected. Some strains failed to grow and others grew only after a long interval in the urea bouillon at pH 7.5 to 7.7. In the more alkaline bouillons growth occurred with less difficulty, but only after an appreciable lag, and certain strains failed to develop in any sample. In all cases, if growth started it progressed rapidly. Control inoculations of urea bouillon sterilised by intermittent steaming were made at the same time, and these invariably yielded prompt growth. It became evident that urea bouillon was converted into a satisfactory medium for *B. Pasteuri* only as a result of heating, and that the beneficial effect of steaming was only in part attributable to the alkaline reaction arising from a partial hydrolysis of urea. The use of different peptones (Bacto, Proteose and Witte), and of meat infusion instead of meat extract for preparing bouillon, did not influence the results.

The next experiments were planned to show whether the beneficial effects of heating urea bouillon arose from an alteration of the urea, the meat extract, or the peptone. Ordinary bouillon, consisting of 1 per cent peptone and 1 per cent meat extract, was prepared in the usual way, and the reaction was adjusted to pH 7.5 by the addition of NaOH. The bouillon was divided into three portions. The first was untreated, the second was adjusted to pH 8.8 by adding Na_2CO_3, and to the third 0.5 per cent commercial ammonium carbonate was added. The three portions were distributed in tubes and sterilised at 22 pounds. A 50 per cent aqueous solution of urea was also prepared. Of this, one portion was sterilised by filtration and a second was autoclaved at 22 pounds. Another urea solution of the same concentration was prepared by weighing the crystals aseptically, dissolving them in sterile water and placing the solution in flowing steam for fifteen minutes. The urea solutions were pipetted aseptically into tubes of the different bouillons in amounts which brought the concentration of urea to 2 per cent in each case. The reaction of all the media was then adjusted to pH 7.8 by adding sterile Na_2CO_3 or H_3PO_4. The different media were inoculated by transferring to each tube a loopful of a faintly turbid suspension of cells in phosphate buffer. Table 1 gives the essential features of one of the experiments in which an incubation temperature of 30°C. was employed, the intensity of growth at twenty-four hours being indicated by the number of + symbols. The table shows that heating the bouillon which had been made alkaline with Na_2CO_3 did not improve the medium. The beneficial effect obtained by the use of ammonium carbonate can therefore be attributed to the presence of ammonia in the medium and not to an alteration of the bouillon constituents produced by sterilisation at an alkaline reaction. The table also shows that heating the urea apart from the bouillon resulted in an improved medium, and that the most drastically heated urea gave the best result. This effect can be attributed to a partial conversion of the urea to ammonium carbonate at the high temperatures. The urea solutions were all tested for cyanates with $AgNO_3$ but the results were negative. These experiments were carried out with several strains, some of which grow more readily than others on media without urea. The results were approximately the same in the case of each strain except one. The latter, an organism which is exceptional in its ability to grow easily on ordinary media, was not influenced definitely by the presence of ammonia. The results of these experiments were confirmed by using agars instead of bouillons. Agar media produced clearer distinctions than bouillons for, in addition to showing the total amount of growth, they gave an indication of the relative number of cells in the inoculum which initiated growth on each medium.

TABLE 1

Growth in 24 hours at 30°C. in media containing 1 per cent peptone, 1 per cent meat extract and 2 per cent urea; pH 7.8

BOUILLON	UREA		
	Filtered	Steamed 15 minutes	Autoclaved
1. Ordinary; pH 7.5 before sterilisation...	–	–	+
2. Adjusted to pH 8.8 with Na_2CO_3 before sterilisation	–	–	+
3. Ammonium carbonate (0.5 per cent) added before sterilisation...........	+	++	+++

It appeared to be certain that the ammonia requirement of the organisms is unusually high. Experiments were accordingly carried out to test the effect of adding ammonium salts to bouillon or agar. In the first tests the media, adjusted to pH 7.8 to 8.2, were made up to contain when completed 1 per cent peptone, 1 per cent meat extract, 2 per cent urea sterilised by filtration, and varying proportions of $(NH_4)_2SO_4$. The last two ingredients were added to the remainder after sterilisation. The tests again demonstrated that growth was considerably delayed in the absence of added ammonia, and they further showed that the addition of 0.2 per cent $(NH_4)_2SO_4$ to the media containing unheated urea permitted growth as readily as did urea bouillon or urea agar sterilised by steaming.

In the succeeding experiments, media containing different concentrations of $(NH_4)_2SO_4$ or NH_4Cl but no urea, were used. The ammonium salts in concentrated aqueous solution were sterilised separately and added to the alkaline media immediately before inoculation. Growth occurred most readily when about 1 per cent of either salt was added, and if the reaction was close to pH 8 the organisms developed almost as quickly as in the usual urea media. The optimum concentration of ammonia was not clearly defined: most of the strains examined appeared to grow with equal facility at any concentration between 0.5 and 2 per cent. The optimum for certain strains which decompose urea slowly appeared to be between 0.5 and 1 per cent of either the sulphate or the chloride. The maximum concentration of ammonia tolerated by the organisms is high, but in attempting to elucidate this factor the results were erratic and inconclusive. Wide differences were obtained with a single strain when different amounts of inoculum were used.

THE EFFECT OF REACTION

Having obtained a suitable medium which is not subject to violent changes in reaction as a result of growth, a study was made of the effects of hydrogen ion concentration. Bouillon with a high buffering capacity, containing 2 per cent peptone, 2 per cent meat extract and 1 per cent NH_4Cl, was used. In order to obtain a medium which would remain clear when made alkaline, the bouillon was prepared by adjusting the reaction to approximately pH 9, autoclaving, filtering, and bringing the reaction back to neutrality, prior to adding the ammonium salt and sterilising. By adding sterile Na_2CO_3 or HCl aseptically to samples of the bouillon, several series of media varying in reaction were obtained. These were inoculated with dilute suspensions of the organisms, and observations were made for the appearance of growth during incubation.

For most strains the optimum reaction proved to be in the region of pH 9, but in the case of the least active urea-decomposing organisms it was nearer to pH 8. The active strains seldom grew at pH values below 7.5, while the inactive types usually produced visible growth at pH 7. In these experiments few strains developed on the acid side of pH 7; but on other occasions a number of strains were observed to grow in media as far from neutrality as pH 6.2 to 6.4, if heavy inoculations were made. The alkaline limit for growth appears to be inconstant even for individual strains: the vigour of growth, the composition of the medium, the temperature, and probably other factors seem to produce variations.

NUTRITIVE AND OTHER ASSOCIATED REQUIREMENTS

The growth of the organisms was tested in a considerable number of media. The most suitable substrates were prepared from bouillon containing comparatively high concentrations of peptone and meat extract. The use of at least 1 per cent peptone is especially important in the case of urea-containing media, in order to prevent cultures from becoming excessively alkaline. Optimal conditions for growth in bouillon were produced by adding 0.5 to 1 per cent NH_4Cl, and adjusting the reaction to between pH 8.5 and 9 after sterilisation. The organisms grew more profusely in this medium than in 2 per cent urea bouillon. They also developed earlier than in urea bouillon, especially if the latter had been allowed to stand for several weeks after preparation, so that its ammonia content was reduced. Alkaline ammonia-containing media cannot be sterilised without deterioration, and urea media are therefore more suitable for general use. The comparative values of ammonia and urea suggest that the latter compound does not serve as an energy source in the metabolism of the organisms. It appears to be impossible, however, to investigate this question satisfactorily with the complex media which were found to be necessary in this work. Söhngen (1909) and Christensen (1910) stated that certain bacteria which decompose urea may utilise energy liberated by the reaction, but their findings were not supported by any decisive proof.

In nature, bacteria of this group seldom encounter media comparable in nutritive value to bouillon, and their ability to grow in poorer substrates was tested. In autoclaved soil, soil extract and dilute peptone solutions (0.1 to 0.01 per cent) the organisms were capable of growing, provided the reaction and the content of ammonia were suitable, but the amount of growth produced was meagre in comparison with that in bouillon cultures. A visible growth was never observed in synthetic solutions which contained glucose or salts of organic acids as carbon sources, and asparagine with the addition of either ammonia or urea as nitrogen sources. No further efforts were made to confirm the findings of Viehoever (1913) and Rubentschik (1925, 1926) that organisms of this group are capable of multiplication in synthetic media, for if bacteria are unable to produce a macroscopic growth in a transparent test solution, evidence of their proliferation obtained by counting methods is generally of uncertain value.

Observations which indicate how bacteria of the Pasteuri group may find in nature conditions suitable for growth were made from experiments in bacterial association. In protein-containing media to which neither ammonia nor urea were added, organisms of this group, while incapable of multiplying in pure culture, were able to proliferate actively in the presence of other bacteria which decompose the protein. Solutions of casein and gelatin (1 per cent) adjusted to pH 7.5 to 7.7 were inoculated with *B. mycoides* and with a small number of cells from a dilute emulsion of a Pasteuri agar culture. Several strains of the urea-decomposing organisms produced, after incubation for some days, a densely turbid growth, distinguishable from the flocculent growth of *B. mycoides*, and by plating on urea agar, the number of viable cells of the former was found to be of the order of 10^{10} per ml. of culture. Growth of the urea decomposers took place more readily if they were inoculated into cultures of *B. mycoides* which had been incubated previously for seven to fourteen days so that the protein decomposition was already well advanced. In the tests with casein and gelatin the strains of *B. Pasteuri* which are especially dependent on an alkaline reaction and a high concentration of ammonia did not initiate growth easily, but these strains proved to be capable of developing on ordinary agar in association with other bacteria, such as organisms of the Coli group. When agar was used, the liquefied medium was inoculated with the urea decomposer, poured into a Petri dish, allowed to solidify, and then inoculated at one point with the second organism. After a period of incubation, the length of which depended on the strain used, colonies of the urea-decomposing bacillus appeared in the agar around and beneath the growth of the other organism, but not on the remainder of the plate. It seems probable that the ability of organisms of the Pasteuri group to grow in the presence of other bacteria depends on the production of ammonia by the latter.

The inability of *B. Pasteuri* to grow in the ordinary bacteriological media adjusted to neutrality has been considered an important diagnostic feature. The Pasteuri group does, however, comprise a series of types varying from the most active urea-decomposing strains which cannot be cultivated in ordinary neutral bouillon, to feebly ureaclastic organisms which grow readily from small inocula. A test with ordinary media does not therefore permit of a clear differentiation of the group, and it is further complicated by variations due to the amount of inoculum and, especially in the case of agar, by the evolution of ammonia from other cultures in an incubator. Neutral one per cent peptone is of greater differential value than bouillon. Of the strains examined in this work only three develop easily in a peptone solution if the medium is lightly inoculated. Milk and potato, probably on account of their reaction, do not support the growth of any strain unless they are inoculated fairly heavily.

THE FORMATION OF NITRITES

In seeking an explanation of the unusually high ammonia requirement, the possibility that the organisms utilise ammonia as a source of energy was considered. Viehoever (1913) has claimed that bacilli of this group are capable of oxidising ammonia to nitrous acid, and that they may multiply to some extent in a solution in which ammonium carbonate constitutes the only source of carbon and nitrogen. Should this claim be substantiated, it would be of more than theoretical interest, for the invariable occurrence of these organisms in farm manure would account for a loss of nitrogen through nitrite formation and subsequent denitrification. Several of the strains used in this work, when cultivated in peptone water containing 0.5 per cent ammonium sulphate, produced appreciable quantities of nitrites as shown by using the Griess-Ilosvay reagents, while other strains did not. The strains which formed nitrites are all nitrate reducers and those which gave negative or doubtful tests do not reduce nitrates. It seemed probable, therefore, that the nitrites were formed by the reduction of traces of nitrates in the medium. There are numerous records of the formation of small amounts of nitrous acid from ammonia or from organic matter by heterotrophic microörganisms, but it should be pointed out that experimental work on this problem is particularly subject to error. The chief difficulties arise from the use of highly sensitive reagents for nitrites, the lack of comparably sensitive nitrate reagents, the frequent occurrence of nitrates as impurities in the ingredients of culture media, and the presence of oxides of nitrogen in the atmosphere, especially in the proximity of Bunsen burners. Without describing the exploratory work, it may be stated that after trying a considerable number of media and after preparing the purest ammonium salts, it was found that strains of *B. Pasteuri*, which readily formed nitrites when tested in the ordinary way in ammonia peptone solutions, could be cultivated at 30°C. for periods up to seven days under conditions permitting of excellent growth, without a trace of nitrous acid being formed. In the final experiments media were used immediately after preparation, the usual flaming of culture containers was omitted, and the cultures were incubated in a stream of purified air. It was concluded that *B. Pasteuri* is incapable of oxidising ammonia. The experiments also suggest that similar work with other heterotrophic bacteria requires the most rigid control.

SUMMARY AND CONCLUSIONS

Most of the organisms which may be placed in the *Bacillus Pasteuri* group require alkaline media containing ammonia. In the case of strains which bring about the most rapid decomposition of urea and are incapable of growing in ordinary neutral media, optimal conditions for growth in bouillon are produced by adding about 1 per cent NH_4Cl and adjusting the reaction to about pH 9. Strains which produce a weaker action on urea are less dependent on ammonia and an alkaline reaction, but few of them grow readily from small inocula on the ordinary bacteriological media. The most suitable substrates for cultivating the organisms contain urea from which the necessary ammonia is formed during sterilization and later by the urease activity of the bacteria. The organisms grow most readily in media containing comparatively high concentrations of peptone and meat extract. A visible growth has not been observed in synthetic solutions. In media containing ordinary bouillon or a protein, the organisms, while incapable of growth in pure culture, may proliferate actively in the presence of other bacteria. The appearance of nitrites in ammonia-containing cultures may be attributed to one or more of several circumstances, but not to an oxidation of ammonia by the organisms.

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A BASAL MEDIUM FOR THE PRIMARY ISOLATION OF PATHOGENS

A QUICKLY PREPARED MEDIUM INCLUDING ACCESSORY GROWTH FACTORS FROM FISH ROE OR QUAHAUGS

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It has long been known that the growth of bacteria on culture media is facilitated by the addition of certain substances, soluble in water, thermostable at 100°C. and readily adsorbed from solution by filtration through paper or cotton.

Dorothy Lloyd (1916) in her description of the growth of the meningococcus designated such growth-promoting bodies as "accessory growth factors;" "accessory growth hormones;" and "vitamine factors." Later, in a report on the cultivation of the gonococcus, Cole and Lloyd (1917) called them "special growth hormones;" "special growth factors;" and "growth hormones." Subsequently, Huntoon (1918) writing of "Hormone" medium, abbreviated the above terms to "growth factors," and "hormones." In this paper we use the phrase—accessory growth factors—for substances having the properties mentioned above. A culture medium containing accessory growth factors is designated as a hormone medium.

An investigation of the possible value of fish tissue in hormone media was prompted by the question whether the fairly rapid putrefaction of dead unpreserved fish during warm weather might be the result of the presence of easily assimilated nutriment—or of accessory growth factors.

The species of fish first used in the investigation was the horned pout or bull head. Three tissues were utilized—muscle, kidney, and roe. The following technique was employed. The tissue was finely ground in a meat chopper, with the exception of the roe, which was forced through a fine sieve. Of each tissue 100 grams were infused in 200 cc. portions of distilled water and placed in the icebox overnight. The next morning the infusions were brought to a boil over a flame and allowed to boil one minute. Each infusion was filtered through glass wool packed in the stem of a funnel. Of each filtrate 8, 20, 40, and 80 cc. portions were then diluted to 200 cc. with distilled water. These dilutions represented approximately 2, 5, 10, and 20 per cent infusions of the original tissue. To 100 cc. of each infusion were added 3.1 grams of dehydrated Difco nutrient agar 1.5 per cent. The infusions were heated to facilitate solution of the agar, and the pH of each adjusted colorimetrically to 7.7 to allow a final pH of 7.3 to 7.4 after autoclaving. A control medium consisting of 3.1 grams of dehydrated Difco nutrient agar 1.5 per cent in 100 cc. of distilled water at pH 7.7 was also prepared.

TABLE 1

Macroscopic growths after twenty-four hours incubation at 37.5°C.

MEDIUMS	2 PER CENT	5 PER CENT	10 PER CENT	20 PER CENT
1.5 per cent nutrient agar, control	No visible growth			
Fish kidney	Slight visible growth in all			
Fish muscle	Poor	Poor	Fair	Fair
Fish roe	Very good	Excellent	Very good	Fair

In conformity with our usual procedure, 15 cc. amounts of these media were distributed into 4-dram glass vials with aluminum screw caps which were fitted with a rubber interliner cut with a cork borer from an automobile inner tube. The lips of the vials were ground even. The use of such vials allows an air- and pressure-tight seal, thus insuring against loss of moisture from media upon prolonged storage. The media were autoclaved at 15 pounds pressure for twenty minutes. Media, solidified after autoclaving, were melted in a bath of boiling water, and poured into Petri dishes. After solidification, the surface of each medium was inoculated, by the streak method, using a beta type of streptococcus. This strain of streptococcus had been transplanted but once from a blood culture to Loeffler's medium. A small loopful of culture from the water of expression was used. After twenty-four hours incubation at 37.5°C., macroscopic growths were as shown in table 1.

The results of the experiment on fish roe medium were quite unexpected. Not only was the growth distributed over all the streaks on the plate, but the size of the colonies equalled that usually obtained on blood-infusion agar. A decrease in growth along with an increase in concentration of roe above 5 per cent was another peculiar feature of this medium.

A series of experiments was then designed to ascertain the optimum concentration of horned-pout roe yielding the best growth-promoting quality; and the value of other species of fish roe in culture medium. Infusions of horned-pout roe were made from 1 to 50 per cent in distilled water, the technique of preparing each infusion and making the finished medium being the same as above. The same strain of organism was used in testing, and the results were approximately the same. In more than 5 per cent concentration, the horned-pout roe showed only a questionable increase in growth-promoting quality. Various species of fish roe, including speckled brook trout, wall-eyed pike, yellow perch, Atlantic salmon, fresh-water smelt, salt-water cusk, mackerel, frozen mackerel, lemon sole, shad and canned shad were used in experiments. All of the various fish roes tried, with the exception of canned shad roe, showed the presence in varying proportion of accessory growth factors. The more mature the roe, the less concentration was needed, and the better was the growth-promoting quality.

From time to time, as different strains of pathogenic bacteria were isolated in routine examinations, they were cultured on the various media. These species included *Staphylococcus aureus*, *N. gonorrhoeae*, *N. intracellularis*, *N. catarrhalis*, *D. pneumoniae* type I and III, streptococci of the alpha, beta, and gamma type, *Cl. Welchii*, *Es. coli*, *Ps. aeruginosa*, *B. anthracis*, and *C. diphtheriae*. All these grew on roe medium with a luxuriance equal to, and usually exceeding, that on blood infusion agar.

Since the supply of mature fish roe is seasonal, we investigated other sources of supply of accessory growth factors. Those tried have been quahaug, soft-shelled clam, sea-scallop, oyster, yolk of hen's egg, and potato. The scallops and oysters were of no definite value; the potato and egg yolk promising, soft-shelled clams fair; and quahaugs excellent.

Since quahaugs can be obtained easily throughout the year, and the quality of the accessory growth factors seems quite uniform, this source of supply has been used extensively in our later experiments. While the optimum percentage infusion is somewhat greater than with roe, being about 20 per cent, the results obtained have been in all respects equal to fish roe medium. The objection to the quahaug medium was that the filtrate appeared always quite colloidal and almost opaque. After autoclaving, considerable sediment was deposited in the bottom of the tubes, and at times the supernatant fluid was too cloudy. Experimentation showed that if the quahaugs were infused in an acid menstrum, the resulting filtrate, after treatment with NaOH, was only slightly opalescent, and the sediment in the finished medium very slight. From experimental tests it was found that the acid infusion yielded a high percentage of accessory growth factors, and gave a clear solution when filtered through glass wool. Our present procedure of preparing the quahaug infusion is as follows:

Medium to large quahaugs are shucked into a coarse sieve, and the juice allowed to drain thoroughly. The quahaugs are then ground fine in a meat chopper. To 990 cc. of distilled water are added 10 cc. of concentrated HCl (C.P.) and 200 grams of finely ground quahaugs. This infusion is placed in a pan, brought to a boil, and allowed to boil for thirty seconds. It is then filtered through loosely packed glass wool. After adjusting the pH to 7.8 or 8.0, the filtrate is again brought to a boil. A second filtration is then made through a glass wool plug in the stem of a funnel. The resulting filtrate should be clear by direct light, with a suggestion of opalescence by reflected light. To each 1000 cc. of infusion are added 31 grams of Difco nutrient agar 1.5 per cent. This mixture is boiled until the agar is dissolved. The pH is adjusted to allow a final pH of 7.4 to 7.5 after autoclaving. Any small amount of sediment formed during autoclaving is allowed to remain in the vials on pouring the plates.

The above mentioned strains of bacteria which had been grown on fish roe medium were also cultured on quahaug medium. The luxuriance of growth was equal to that obtained on fish roe medium, and in many instances superior.

We have also had excellent results in using the quahaug medium for primary isolation of molds from skin and lung lesions.

The following media have been used in preference to Brown's (1919) veal infusion agar for the identification of streptococci of the alpha, beta, and gamma type.

Quahaug infusion (as above), 20 per cent	1000 cc.
or	
Fish roe infusion (as above), 5 per cent	1000 cc.
Witte's peptone	10 grams
NaCl	5 grams
Agar—Merck's reagent	15 grams

The medium is brought to a boil, and the boiling continued until the agar is dissolved. The pH is adjusted to 7.8. The medium is placed in a 1000 cc. leveling bulb fitted with a rubber tube and a pinch cock, and allowed to steam in the Arnold until the supernatant fluid is clear. The sediment is drawn through the rubber tube by releasing the pinch cock. The pH of the medium is adjusted to 7.7 (if necessary) to allow a final pH of 7.4 after autoclaving. The medium is tubed in screw-capped vials in 10 cc. amounts and sterilized at 15 pounds pressure for twenty minutes in the autoclave. After melting the medium and cooling to 45°C., 0.5 cc. of sterile defibrinated sheep blood, and the material to be tested, are added to each vial. After thorough mixing, the plate is poured. Using these media, with Brown's veal infusion agar as a control, we have run over 200 cultures from throat swabs and surgical swabs. In all cases, determination of type of streptococcus was made in twenty-four hours, without storage in the ice-box, and in most instances in ten to eighteen hours. The cultures have been made directly from the swabs. In no instance has the type of organism differed in either medium from the control. The rapidity and sharpness of the reaction of hemolysin is much superior on the quahaug or fish roe medium, using defibrinated sheep blood, to that obtained on Brown's standard medium using the same kind of blood.

Both fish roe and quahaug infusion may be prepared with dehydrated Difco nutrient broth added, and an excellent liquid medium equalling "hormone" infusion broth obtained.

Dehydrated North's gelatin agar has been added to fish roe and quahaug infusions, but the growth-promoting quality of these media has not been uniform. We have used an infusion of quahaugs, part of which contained North's gelatin agar, the remainder Difco nutrient agar 1.5 per cent as a control. Culturing the same strains of organisms, we have observed, at times, a luxuriance of growth decidedly in favor of the North's gelatin agar. In other experiments, no growth was obtained on North's gelatin agar when excellent growth was observed on nutrient agar 1.5 per cent. Since we have used three different batches of North's gelatin agar in our experiments, we are now retesting these to determine if the discrepancy in results is due to a variable factor in the North's gelatin agar. To-date various batches of Difco nutrient agar 1.5 per cent have shown quite uniform results in growth-promoting quality, when reinforced with the above infusions.

CONCLUSIONS

1. Fish roe and quahaug have been shown to be good sources of accessory growth factors. .
2. A quick method of preparing hormone agar and broth media from these substances has been given.
3. The advantages of these ingredients as used with Brown's technique in determining types of streptococci have been notable.

To Dr. E. H. Buttles I am indebted for his constructive criticism and assistance in obtaining pathological material, and to Dr. A. B. Soule, Jr., for the photographs.

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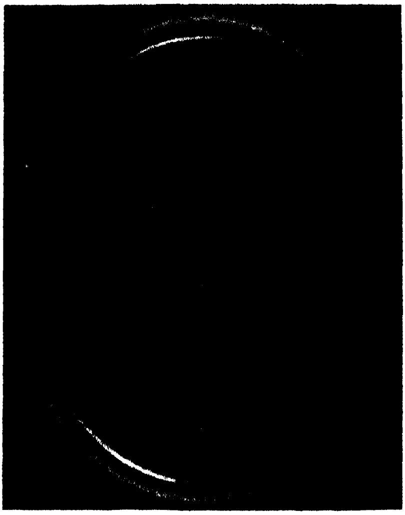
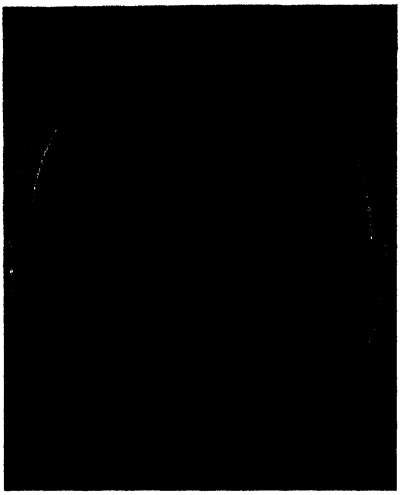


A

Material Swab from throat of patient with clinical scarlet fever
Petri dish. 75 by 10 mm
Culture medium 20 per cent Quahaug infusion plus Difco nutrient agar 1 5 per cent
Incubation Aerobic; 18 hours at 37 5 C
Small colonies Beta streptococci



B

Material Same as A
Petri dish 75 by 10 mm
Culture medium Control. Difco nutrient agar 1 5 per cent.
Incubation. Identical with A

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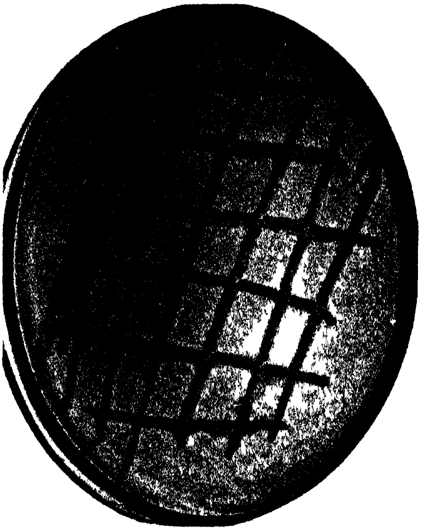
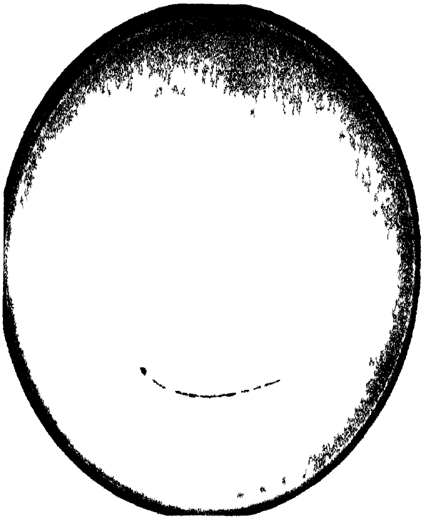


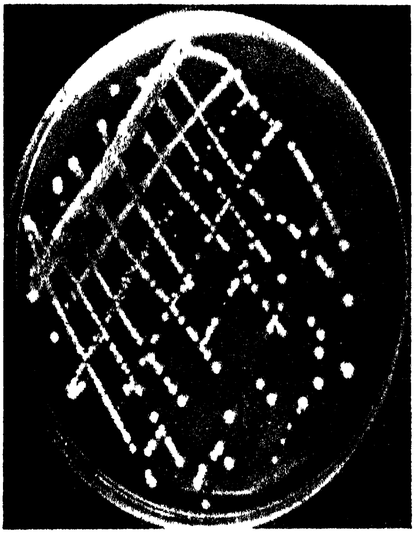
Material. Swab from throat of patient with clinical diphtheria.
Petri dish. 100 by 15 mm.
Culture medium. 20 per cent Quahaug infusion plus Difco nutrient agar 1.5 per cent.
Incubation. Aerobic, 18 hours at 37.5°C.
Remarks. *C. diphtheriae* outgrew other microorganisms to the extent of resembling a pure culture.



B

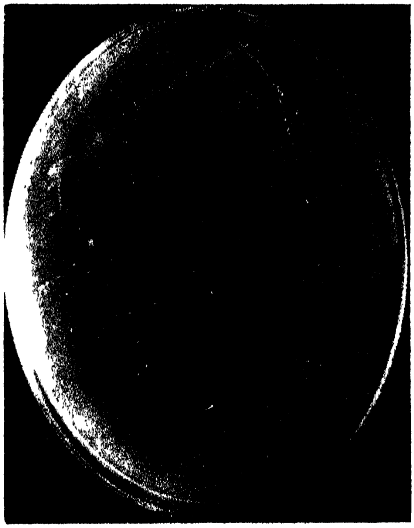
Material. Same as A.
Petri dish. 100 by 15 mm.
Culture medium. Control. Difco nutrient agar 1.5 per cent.
Incubation. Identical with

W. E. James: Basal Medium for Primary Isolation of Pathogens)



A

Material: Exudate from thigh of patient with gunshot wound.
Petri dish: 75 by 10 mm.
Culture medium: 20 per cent Quahaug infusion plus Difco nutrient agar 1.5 per cent.
Incubation: Anaerobic in Varney jar. Sixteen hours at 37.5 C.
Colonies: *Cl. Welchii.*



B

Material: Same as A.
Petri dish: 75 by 10 mm.
Culture medium: Control. Difco nutrient agar 1.5 per cent.
Incubation: Identical with A.

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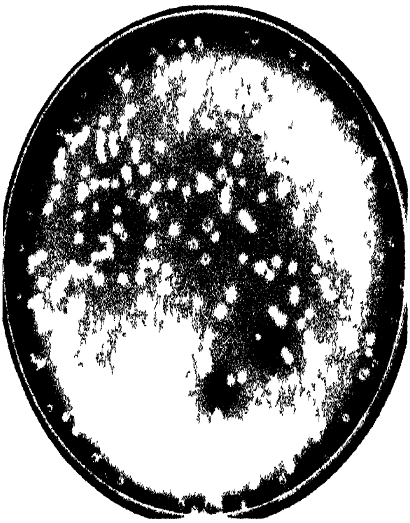
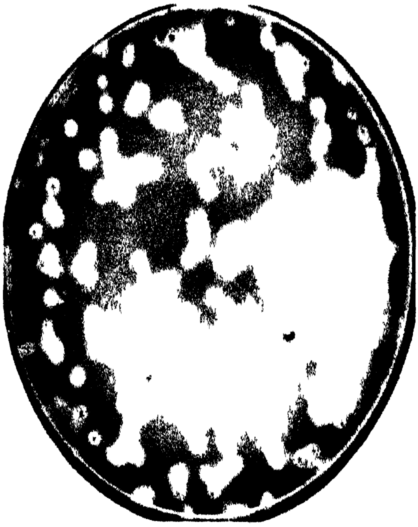


Material Alpha streptococcus obtained from a bacteremia
Petri dish 100 by 15 mm
Culture medium 5 per cent mackerel roe infusion plus Witte's peptone NaCl 1.5 per cent agar and 5 per cent defibrinated sheep's blood
Incubation 12 hours at 37.5 C Subsequently 1 hour at room temperature



B

Material Beta streptococcus direct from a throat swab
Petri dish 100 by 15 mm
Culture medium 5 per cent mackerel roe infusion plus Witte's peptone NaCl 1.5 per cent agar and 5 per cent defibrinated sheep's blood
Incubation 12 hours at 37.5 C

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EVALUATION OF SOME FACTORS INFLUENCING THE PHENOL RESISTANCE OF STAPHYLOCOCCUS AUREUS[1]

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This study was undertaken to find the most important causes of the large day-to-day variation in resistance to phenol of cultures of *S. aureus*, and to determine the accuracy to be expected from determinations of this resistance, and thus from disinfectant tests.

Chick (1910) working with *S. aureus* under apparently constant conditions, reported a variation in death-rate constant from 0.085 to 0.320 in a series of tests made on different days. Miller (1920) found the chief difficulty in his work with the same species to be this day-to-day variation in the resistance of the organism. Knaysi and Gordon (1930) stated that with the same strain under identical conditions, it is difficult to obtain comparable results in disinfectant and death-rate tests. Almost anyone who has tried to make careful death-rate determinations with bacteria has experienced the same difficulty. One is led to believe that, in spite of the care exercised in much of this work, there have been one or more inadequately controlled variables responsible for these changes in the resistance of a single strain.

Generally speaking, the accuracy to be expected from quantitative procedures in bacteriology is not well known. The statement most nearly covering the technic of this work is given by Cohen (1922) who found a probable error of 2 or 3 per cent under the most ideal conditions in death-rate determinations, and as high as 10 per cent in most of his tests.

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[1] Based on a thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Pennsylvania.

All conditions in the disinfectant test are ordinarily specified, and apparently only deviations from specifications can be important in causing variation. The problem then resolves itself into a series of estimates of the magnitude of the errors to be expected from probable deviations from the prescribed conditions.

To evaluate these errors, a careful examination of the technic was first necessary, to prove the dependability of the methods used, and of the individual manipulations of the author. The variable conditions studied could be divided into those having an influence only on the determination, and those having an influence on the resistance of the culture. The former included time of subculturing, concentration of disinfectant, and of cells, and temperature of disinfection. The latter included volume of inoculum, age of culture in hours, and in transfers from agar, and incubator temperature. In all cases, emphasis was placed on small deviations which might conceivably occur through only slight carelessness in an actual disinfectant test.

METHODS

All pipettes were checked, retaining only those with errors of less than 0.5 per cent. Dilution bottles were filled from a pipette after sterilizing, within twelve hours of their use. All temperatures were read to within 0.1°, using thermometers recently checked against an instrument bearing a Bureau of Standards certificate.

Because of its importance in disinfectant testing, and its known high resistance, the Food and Drug Administration strain of *S. aureus*, known as "A 209," was used. Its resistance to phenol was determined both by the survival-time method and by the death-rate method. The former determinations were made according to the description of Ruehle and Brewer (1931) for the phenol coefficient determination, against 1:80 phenol at 37°, making subcultures every fifteen seconds.

Death-rate determinations were based on plate counts, made according to the Standard Methods of Water Analysis (1933) save that the volume put into one plate was one-fifth of the pipette volume, from 0.2 to 2.0 ml., and five plates were made in parallel for each count. All death rates were determined against 0.60 per cent phenol, calculated on the basis of the total volume after adding the culture, rather than before as is done with the survival-time determinations. The time period over which the death rate was measured was uniformly twenty minutes, starting about one-half minute after inoculation. This time was found to avoid the induction period in the death process. The phenol solution was buffered at pH 6.8 with 0.01 molar phosphate.

Death-rate constants were calculated from the formula

$$k = \frac{2.3026}{t} \log \frac{A}{B}$$

where A and B are the initial and terminal populations, and t is the time in minutes. This constant is calculated simply as an aid in comparing the data of the paper, which include only death rates under such similar conditions that the question of whether or not they really represent the logarithmic form of death implied by the equation can be ignored.

RESULTS

Since the results include over 400 death-rate and survival-time determinations, they cannot be presented fully. Most of them are summarized only. Unless specially indicated, all statements in these summaries are based on at least five complete parallel experiments.

The media used in this study are sufficiently well suited to the organism so that plate counts agreed with the dilution method of estimating population, the latter estimates being obtained from the procedure of Halvorson and Ziegler (1933). Five comparisons of the two methods gave an average ratio of 97.8 per cent between the two estimates.

Under the conditions of the study, phenol carried over into the survival culture has no bacteriostatic effect on uninjured cells, since 0.16 per cent is required to prevent visible growth in broth, and less than 0.08 per cent in agar does not decrease the forty-eight-hour plate count.

Under the conditions of the study low osmotic pressure is not a factor in causing death of the bacteria, for in tests using distilled water to replace the phenol the plate count increased over five times in twenty-four hours.

The standard deviation of 800 consecutive sets of plate counts of this study averaged 4.6 per cent of the mean count; the probable error averaged 3.1 per cent. The counts were distributed approximately as demanded by the Poisson series.

The standard deviation of both parallel survival-time and death-rate determinations on five different portions of a single culture was about 7 per cent, and on five parallel cultures was about 10 per cent. In death-rate determinations without a buffer, on five portions of one culture a standard deviation averaging 12.6 per cent was found. It is obvious from this that two cultures must differ by at least 10 per cent in resistance before one can be sure that the difference is real, and not due to random errors in the technic of the test.

The relation found between phenol concentration and survival time in thirty-three determinations is best expressed by the equation

$$1.617 = 7.7 \log c + \log t$$

where c is the concentration of phenol in per cent, and t is the time in minutes. From this equation, a 2-per-cent change in concentration from 1:80 phenol would make a difference of about 2 per cent in survival time. In the death-rate determination, a difference of 0.02 per cent from 0.60 per cent was found to make a difference of 17 per cent in the constant.

At 35° the survival time averaged 200 per cent and the death rate 60 per cent of that at 37°; and at 39° the former was 60 per cent and the latter 140 per cent of that at 37°.

Doubling or halving the concentration of cells in the death-rate determination made a difference of about 17 per cent in the constant. Doubling or halving the inoculum volume made a difference of not more than 10 per cent in the survival time.

Table 1 shows the results of determinations of the resistance of cultures at two-hour intervals from twenty to twenty-eight hours after inoculation. It is seen that even a difference of two hours in the age, considered permissible by Ruehle and Brewer (1931), produces an appreciable difference in death rate. The corresponding difference in survival time is appreciable only when subcultures are made at short intervals. In no case would two hours difference in age have affected a significant result with subcultures made at five-minute intervals, although a four-hour difference would have done so.

In this work it was noted that a marked change in the character of the culture took place somewhere around the twentieth daily transfer in broth. At this time the fragile pellicle became white, instead of the usual cream or yellow. At the same time there was a considerable increase in the proportion of white colonies developing on agar plates. Smears did not reveal any change in morphology, but no fermentation studies were made. Pinner and Voldrich (1932) reported this change of *S. aureus* to an albus form as frequent with daily transfers in broth.

TABLE 1

Relation between age of culture in hours, survival time, and death rate

EXPERIMENT	AGE OF CULTURE	SURVIVAL TIME	DEATH-RATE CONSTANT	
			Numerically	As per cent of constant for 24-hour culture
	hours	*minutes*		
February 11, 1934..........	20	7.0	0 0914	115.4
	22	7.5	0.0854	107.9
	24	7.5	0.0792	100.0
	26	9.0	0.0733	92.6
	28	11.25	0.0692	87.4
Average of five consistent experiments.......	20			122.0
	22			111 2
	24			100 0
	26			93.2
	28			85.9

In an attempt to demonstrate a change in resistance accompanying this color change, four series of daily transfers were started ten days apart, and resistance determinations were made daily on each member of the series for five days. The results appear in table 2. In general the culture nearest the agar slant gave the highest death rate, but otherwise the results show no definite relation between color and resistance. However, it is probable that such a difference might have been found if strains originating in single white and yellow colonies had been examined.

TABLE 2

Relation between age of culture in transfers from agar, survival time, and death rate

TRANSFERS FROM AGAR	SURVIVAL TIME	DEATH-RATE CONSTANT	
		Numerically	As per cent of constant for 13 to 17 generations
	minutes		
3	7.0	0.0635	113.7
13	7.0	0.0558	100.0
23	8.25	0.0451	81.1
33	8.0	0.0457	82.0
4	5.0	0.0706	121.9
14	6.75	0.0579	100.0
24	8.75	0.0561	96.9
34	5.75	0.0700	121.0
5	9.25	0.0568	106.9
15	9.5	0.0531	100.0
25	9.0	0.0600	113.0
35	9.0	0.0597	112.3
6	5.75	0.0608	90.5
16	5.5	0.0672	100.0
26	6.75	0.0506	75.3
36	7.5	0.0570	84.8
7	5.25	0.0707	110.3
17	5.0	0.0641	100.0
27	7.75	0.0596	92.9
37	7.5	0.0583	91.0
Mean for 3 to 7 transfers			108.7
Mean for 13 to 17 transfers			100.0
Mean for 23 to 27 transfers			91.8
Mean for 33 to 37 transfers			98.2

There has been much written on the influence of the incubator temperature on plate counts, the bulk of which like Breed and Pederson (1933) deals with such mixed flora as those obtained from that of milk where different species have widely different optimum temperatures. In this study, table 3 shows that incubator temperatures ranging from about 24° to 38° had no detectable effect on the plate counts, although low temperatures allowed only very small colonies to develop in forty-eight hours. Naturally this observation refers only to a pure culture study of *S. aureus*, and possibly to that of other species as well.

Chick (1908) reported that *E. typhi* cultures grown at 42° were more resistant than those grown at 37°, and also (1910) that the same was true for *S. aureus*. However, the magnitude of the effect for temperature differences likely to be met in the ordinary incubator could not be demonstrated, although it became clear that it was an important cause of early trouble in the present work. Table 4 gives the results of experiments on parallel cultures inoculated from the same tube, but incubated at different temperatures between 34.5° and 39.6°. For better comparison of the data, the table includes only values interpolated between the actual observations to correspond to even temperatures 1° apart. It is evident that there is a large change of resistance with this species when the incubator temperature is changed as little as 1°.

TABLE 3

Relation between incubator temperature and plate count of S. aureus

MEAN INCUBATOR TEMPERATURE DURING 48 HOURS	MEAN PLATE COUNT	STANDARD DEVIATION OF COUNTS
23.7°	133.8	3 8
29.6	133 2	5 7
35.3	135.0	5.0
36.3	131.6	5 9
36.4	133.6	2 5
38.0	136.6	5.8
38.8	134 5	3 0
Mean..................	134.0 ±0.6	

When incubated above about 36.5° all broth cultures were of the "R" type, with flocculent turbidity and pellicle in twenty-four hours, while below this temperature the turbidity was uniform, although no difference in colony form was observed. When incubated at 37° or above, the pH of a twenty-four-hour culture was high, the highest recorded being 7.4, 0.6 unit above that of the sterile broth. Cultures at temperatures above 37° gave lower plate counts. All this evidence points to some fairly definite change in the culture taking place as the incubator temperature is raised above 37°.

TABLE 4

Relation between temperature of incubation and resistance of culture

(Values interpolated between actual observations ranging from 34.5 to 39.6°)

TEMPERATURE	SURVIVAL TIME	DEATH-RATE CONSTANT	
		Numerically	As per cent of value at 37°
	minutes		
36.0	8.5	0.0788	153.3
37.0*	11.25	0.0514	100.0
38.0	14.0	0.0416	81.0
36.0	9.0	0.0675	140.7
37.0	11.5	0.0467	100.0
38.0	13.75	0.0364	78.0
36.0	10.0	0.0622	115 1
37.0*	11.25	0.0540	100.0
38.0	12.5	0.0485	89.7
36.0	5.75	0.0489	108.7
37.0*	7.5	0.0450	100.0
38.0	8.5	0.0388	86.3
36.0	6 5	0.0637	109.6
37.0*	8.25	0.0581	100.0
38.0	9.75	0.0528	90.0
Mean value at 36.0			125.9
Mean value at 38.0			85.0

* Direct observation, not interpolated.

CONCLUSIONS

Any small deviation from the prescribed conditions for survival time or death-rate determinations will produce a difference in the apparent resistance of the culture. These include deviations in concentration of disinfectant and of cells, and in the temperature of disinfection. The first and last named will produce large errors for small deviations likely to occur through slight carelessness.

A two-hour difference in age of culture or a 1° difference in incubator temperature will cause an important difference in the actual resistance of the culture. The latter error seems most likely to have been responsible for most of the day-to-day variation reported in the literature, in view of the notorious faults of the usual incubator, and the general use of ordinary uncalibrated thermometers in incubators.

Survival times and death-rate constants can be determined with a standard deviation of not more than 7 per cent on parallel portions of the same culture. The variation between broth cultures inoculated and incubated together and in every way parallel is enough to increase this to about 10 per cent.

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COAGULASE AND HEMOLYSIN TESTS AS MEASURES OF THE PATHOGENICITY OF STAPHYLOCOCCI[1]

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Except in the case of abscesses and certain skin infections, early studies of staphylococci furnished no definite information concerning their relation to disease. The term "skin cocci," used to designate albus varieties found in the skin, was in common use and even to-day persists in such trinomials as "*Staphylococcus epidermidis albus.*" On the other hand, it was recognized early that cultures from pyogenic foci usually revealed pigmented types. This gave rise to a separation of the species into varieties based on their pigment production. It was generally considered that the white strains were not pathogenic. In this paper we will outline methods which we consider of value for the differentiation of pathogenic from non-pathogenic staphylococci.

Many authors have shown that *Staph. albus*, and many of the intermediate-colored varieties, could be derived from *Staph. aureus.* Pinner and Voldrich (1932) also demonstrated that, of the strains which they examined, the yellow were more pathogenic than the white. Duran-Reynals (1933) showed that the albus variants differed from aureus parent strains in hemolysin production and the liquefaction of gelatin. He also found that all the very invasive strains were of the aureus variety while about 82 per cent of his non-invasive strains were of the albus variety.

While a number of investigators have studied the pathogenicity of staphylococci, the results have been somewhat confusing as the following survey of the literature will demonstrate.

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It is well known that *Staph. aureus* produces a toxin which, under usual cultural conditions, is constant for the strain (Panton and Valentine, 1932). That this may not always be true was shown by Bigger (1933) who demonstrated that the lysin-producing power may vary from time to time. He suggested that this was due to the presence of both lysogenic and non-lysogenic strains in his cultures, the relative overgrowth of one or the other of which led to alterations in the lysogenic power of the whole culture. Most authorities consider that the outstanding feature of a staphylococcus toxin is its hemolysin (staphylolysin) and, on this basis, Burnet (1929) introduced a method of titrating the toxin which was based on its hemolytic activity. He showed that, using rabbit erythrocytes, the hemolytic titre of a toxin was proportional to its toxicity for rabbits. This was confirmed by Panton and Valentine (1932) and Dolman (1932), who stressed the fact that the hemolytic titre may not be reliable with the cells of species other than rabbits. Panton and Valentine concluded that the human and rabbit hemolysins were distinct.

Parker (1924) considered the hemotoxin to be distinct from the rabbit lethal factor and this view was confirmed by Burky (1933) who observed that one of his strains lost its hemolytic power coincident with an increase in its lethal factor. This suggested that while the hemolysin and the rabbit lethal factor are closely related, some factor other than hemolysis may be responsible for the pathogenic effects of certain strains. Burky described three strains. The first was strongly hemolytic and killed rabbits within 24 hours with a general toxemia but no abscess formation. The second strain was only moderately hemolytic and the rabbits either survived or died in a week with multiple abscesses and loss of weight. The third strain was not hemolytic and had no effect on rabbits.

Jones (1930) produced dissociants of *Staph. albus* which were either hemolytic and non-proteolytic (non-gelatinolytic?) or non-hemolytic and proteolytic (gelatinolytic?).

The parallel between the hemolysin content of staphylococcus toxin and the amount of skin-necrosing toxin present was demonstrated by Panton (1932) and Panton and Valentine (1932). Dolman (1932) showed that the heat lability of the dermonecrotic property is marked and closely corresponds to that of the hemolytic property for rabbit erythrocytes. Nicolle and Cesari (quoted by Burnet, 1929) found that their killing toxins also produced marked skin lesions when injected subcutaneously. On the other hand, Weld and Gunther (1931) concluded that the necrotoxin was distinct from the hemotoxin or the leucocidin and Burnet stated that filtrates having no skin-necrosing power may be active hemolysins but that no skin-active filtrates lacking hemolysin had been reported.

Panton and Valentine (1932) showed that, on the basis of 22 strains, the leucocidin was distinct from the hemolysin and divided staphylococci into the four following types based on these two factors:

GROUP	PATHOLOGIC CHARACTERS	LEUCOCIDIN	HEMOLYSIN
1	Produces severe lesions	Strong	Weak
2	Boils and carbuncles	Strong	Strong
3	Sycosis	Weak	Strong
4	Mainly saprophytic	Weak	Weak

This classification was also used by Panton (1932). These authors found that the leucocidin was related to the acute killing toxin. But according to Weld and Gunther (1931), strains having negative hemolysin reactions but positive leucocidin had no lethal effect on rabbits. Julianelle (1922) showed that the hemolytic titre for horse red cells did not correspond to leucocidin production but his comparison may not be of value, because, as previously mentioned, only the red cells of rabbits have been shown to be reliable in studying the toxicity of staphylococci. Finally, Pike (1934) showed that leucocidin differed from the substance which had a depressing action on phagocytes and stated that leucocidin may be identical with hemolysin. On the basis of Van de Velde's experiments, he concluded that leucocidin was not a primary cause of virulence. In this connection, it is of interest that Gay and Oram (1933) found that the most potent streptococcus leucocidin might be given in relatively large amounts to rabbits without obvious effect.

In a very complete study of these phenomena, Burnet (1930) showed that the presence of small amounts of potassium and magnesium were necessary for, but that calcium was actively inhibitory to, hemolysin production. When the calcium was precipitated by the use of oxalated medium, the yield of hemolysin was identical with that produced by growth in an atmosphere of 20 per cent CO_2 (which is optimal for hemolysin production). Growth in oxalated medium gave rise to variants which produced hemolysin in the absence of CO_2. These variants retained their specific susceptibility to special phage. The hemolysin produced by the albus variants showed all the properties described for staphylococcal toxin. For these reasons, Burnet concluded that the various toxins were manifestations of one common toxin. A similar view was presented by Burnet and Freeman (1932), Gengou (1930) and Weld and Gunther (1931).

In dealing with food-poisoning strains, Jordan (1931), Dack, Jordan and Woolpert (1931) and Jordan and Hall (1931) found that both albus and aureus varieties were among those yielding toxic filtrates. Burnet (1930) reached a similar conclusion, and Panton, Valentine and Dix (1931) showed that the toxin production of *Staphylococcus aureus* appeared to be independent of the pigment which had neither toxic nor antigenic properties and which was of the nature of a carotin. Panton and Valentine (1932) demonstrated that there was no association between cultural characters and toxin production, one of their non-toxic strains giving "typical cultural reactions." From these observations, and from the experimental data illustrated in figure 1, it is clear that the color of any culture is not a reliable guide to its toxicity.

Granted that staphylococcal toxins are often hemolytic, there is conclusive evidence in the literature cited of the existence of toxic non-hemolytic strains. Pinner and Voldrich (1932) demonstrated that the golden yellow strains which they studied produced hemolysis, usually liquefied gelatin, coagulated citrated plasma and produced alkali. Since hemolysis and coagulase activity tests of staphylococci have been shown to roughly parallel the pathogenic effects on rabbits (Pinner and Voldrich, 1932 and Daranyi, 1926), we included both tests in a study of the toxicity of this species. The test for alkali production was not as simple as that for coagulase activity and required several days for its determination. For this reason it was temporarily abandoned in favor of the test for coagulase activity.

We were particularly interested in the coagulase test because it had been overlooked by most writers on the subject, particularly English-speaking ones. Burnet (1930) referred to it as "Staphylokinase" and mentioned the fact that, after three hours incubation, only the aureus types showed coagulation but that the albus types showed clotting when left over night. It was interesting to note that rabbits killed by the acute killing toxin did not show intravascular thromboses and the heart blood clotted in the normal time (Burnet, 1929).

Gross (1933) called attention to the fact that, in operations during staphylococcic diseases, for example in surgical interventions on carbuncles or on osteomyelitis, blood coagulation was frequently accelerated. He pointed out that this observation was reported as early as 1908 by Much, who showed that the coagulating power was most pronounced in *Staph. aureus* infections. He mentioned other authors who found that only the pyogenic staphylococci possessed the coagulating power. He was able to confirm the fact that, to a certain extent, the test permits a classification of pathogenic and saprophytic staphylococci. An anti-coagulase was described.

Using hemolysis of rabbit blood agar, coagulation of citrated rabbit blood and coagulation of milk, with or without peptic digestion, and comparing these reactions with the effect of subcutaneous injection of the strains into rabbits, Daranyi (1926) divided his "more than 30 strains" into the following groups:

GROUP	MILK	PLASMA COAGULASE	HEMOLYSIS	RABBIT INOCULATION	CLASSIFICATION
1	Negative	Negative	Negative	No pus	Saprophytic
2	Positive	Negative	Positive	Pus in 6–10 days	Saprophytic
3	Positive*	Positive	Strong	Pus in 2–3 days, with focal necrosis	Parasitic

* Often peptonizing.

In order to test the relative importance of the hemolysis and coagulase-activity tests, we made comparative tests on over 5,000 strains using human plasma for the coagulase test and oxalated rabbit blood agar for the hemolysin test. This exten-

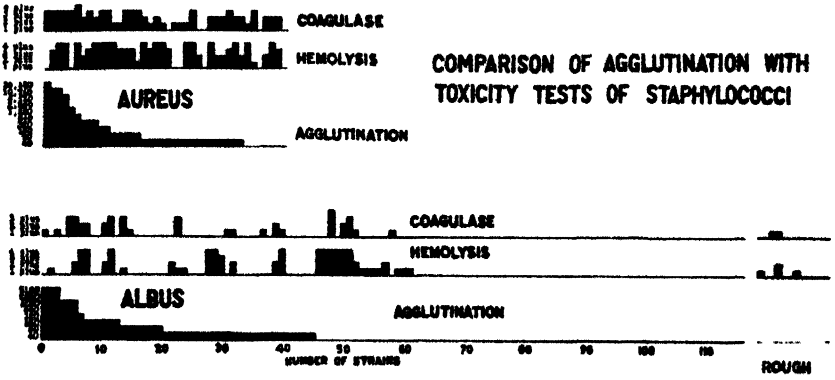

FIG. 1. COMPARISON OF TOXICITY WITH AGGLUTINABILITY

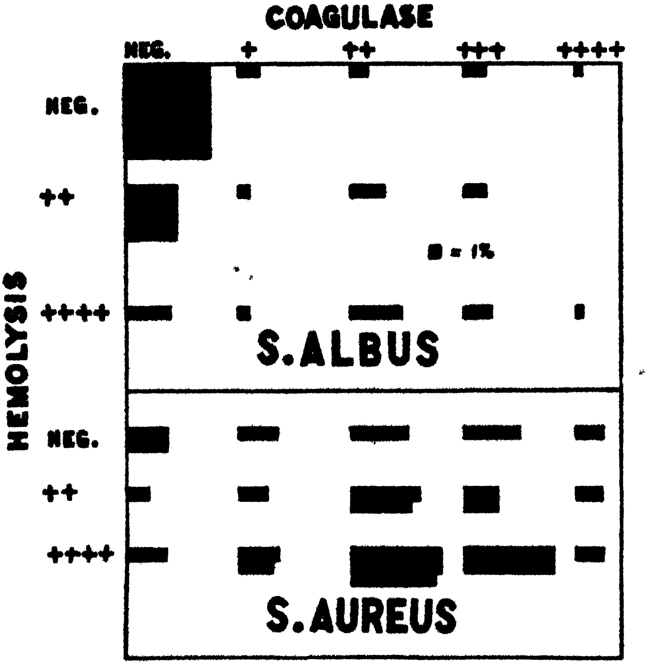

FIG. 2. COMPARISON OF HEMOLYSIS AND COAGULASE REACTIONS OF STAPHYLOCOCCUS AUREUS (368 STRAINS) AND STAPH. ALBUS (885 STRAINS)

sive comparison was considered desirable since much of the previous contradictory evidence was thought to be due to the use by each author of a limited series of cultures. Much of the confusion appears to have arisen from the fact that although most strains contain these toxic fractions in parallel quantities, they may be present in unequal amounts in certain strains. By studying a large series of strains we found that, while the hemolysis and coagulase-activity tests usually gave parallel reactions, instances occurred in which one was strongly positive and the other entirely negative (fig. 1). In some cases where no hemolysin was detected, the strains possessed considerable coagulase

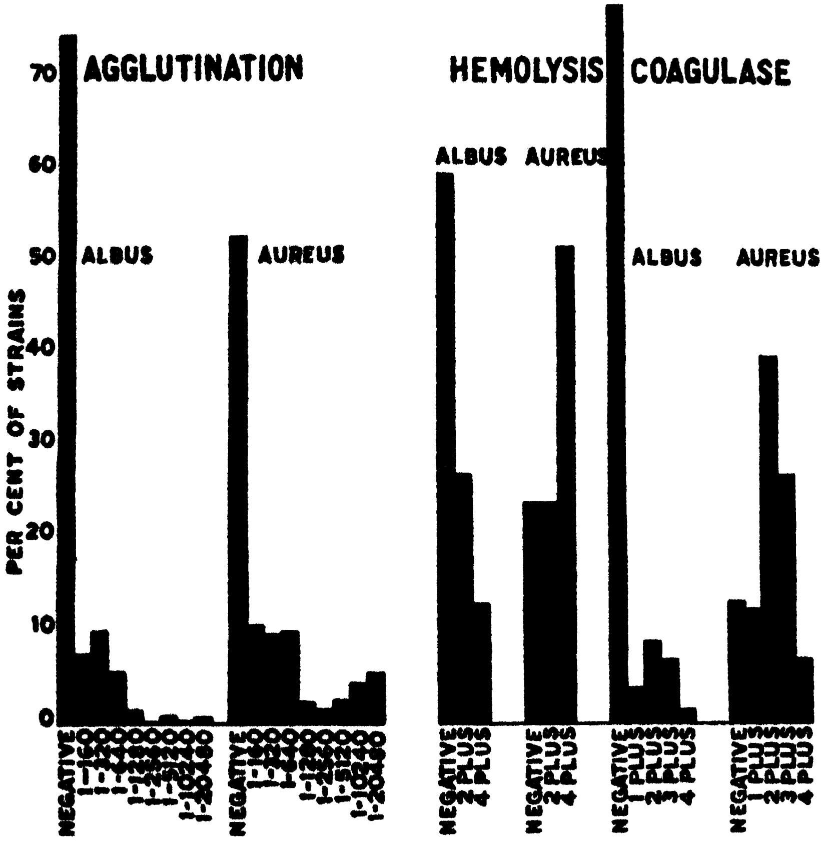

FIG. 3. COMPARISON OF SEROLOGICAL REACTIONS OF STAPHYLOCOCCI (368 AUREUS AND 885 ALBUS STRAINS)

activity. Other strains were hemolytic but did not coagulate plasma. The strains which were lacking in one of these factors, were selected for animal inoculation.

For the coagulase test, one loopful of the twenty-four-hour culture on solid medium was mixed with 0.5 cc. of citrated human plasma and incubated for three hours at 37°C. The tubes were inspected at the end of one-half hour, one hour, two hours and three hours.

For the purpose of this study, porcelain white and creamy white strains were considered as *Staph. albus* while lemon yellow and golden yellow strains were considered as *Staph. aureus.*

The data recorded in figure 3 illustrate the larger number of positive hemolysis, coagulase and agglutinin reactions encountered in strains of *Staph. aureus* as compared with *Staph. albus.* The correlation of hemolysis with coagulase activity tests is shown in figure 2. The majority of albus strains gave weak or negative hemolysis and coagulase reactions while aureus strains are usually positive to both tests. The distribution of these positive reactions, particularly among the aureus strains, suggests that these two reactions may represent independent properties of various strains of staphylococci. Coagulase is more consistently a property of *Staph. aureus.* The difference between the agglutination reactions and hemolysis tests of the two types of staphylococci is less pronounced.

Animal inoculation was made by injecting 5 cc. of an 18-hour culture of each monophasic toxic strain and non-toxic control strain in brain-heart infusion intravenously into healthy rabbits. Pinner and Voldrich (1932) had shown that 5 cc. of a broth culture of albus strains did not produce demonstrable lesions or apparent disease in rabbits. The results are recorded in tables 1, 2, 3, and 4 and figure 4. As previously suggested, the aureus strains were more frequently pathogenic than the albus strains. Consequently we have separated them in the tables in order to determine whether there is any significant difference between representative strains of these two varieties. It is important to re-emphasize that the strains selected for animal inoculation were selected from a large number of toxic strains only because they lacked certain toxic phases. The results recorded in table 1 show that coagulating non-hemolytic albus strains are nearly

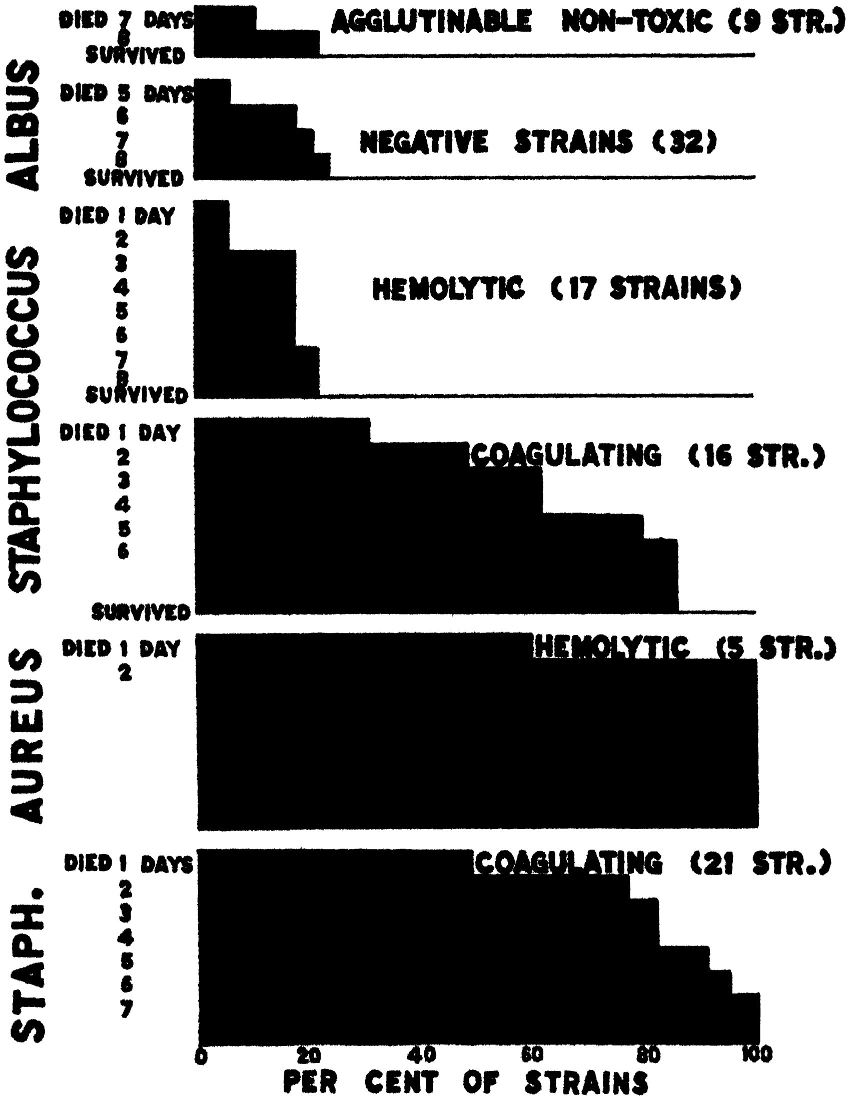

Fig. 4. Comparison of Hemolysis and Coagulase Activity Tests (and Some Agglutination Reactions) with Pathogenicity for Rabbits of 100 Strains of Staphylococci

always pathogenic for rabbits when injected intravenously, but that non-coagulating hemolytic albus strains are nearly always

TABLE 1

Injection of 5 billion live Staph. albus intravenously into rabbits

STRAIN NUMBER	HEMOLYSIS	COAGULASE*	AGGLUTINATION	EFFECT ON RABBIT	REMARKS
3212	0	+++	0	D 5	Coagulating non-hemolytic strains
3286	0	++	1:1280	D 2	
2949	0	++	1:640	D 1	
3189	0	++		D 1	
3190	0	++	1:640	D 1	
2832	0	+	1:10240	D 6	
3222	0	+	0	D 2	
2953	0	++++	0	Survived†	SR strain
3175	0	+++	0	Survived	
4526	0	+	0	D 1	
4529	0	+	0	D 2	
4577	0	++++	1:320	D 3	
4592	0	+++		D 5	
4643	0	++	0	D 5	
4806	0	++		D 3	
4816	0	++++	1:640	D 1	
4593	++++	0		Survived	Hemolytic non-coagulating strains
3207	++++	0	1:160	D 7	
2891	++++	0	0	D 3	
3252	++++	0	0	D 3	
3284	++++	0	0	D 1	
2975	++++	0	0	Survived	
3259	++++	0	0	Survived	
3270	++++	0	0	Survived	
3271	++++	0	0	Survived	
3326	++++	0	0	Survived	
3338	++++	0		Survived	
4562	++++	0	0	Survived	
4613	++++	0	0	Survived	
4681	++++	0	0	Survived	
4693	++++	0	1:320	Survived	
4735	++++	0	1:640	Survived	
4775	++++	0	0	Survived	

* Using human plasma.

† Survived 10 days.

D, died (number following symbol indicates number of days after inoculation).

non-pathogenic for rabbits. As far as these experiments were concerned, there was no significant difference between the agglutinable and the inagglutinable strains. Regardless of their

TABLE 2

Injection of 5 billion live Staph. albus intravenously into rabbits

STRAIN NUMBER	HEMOLYSIS	COAGULASE*	AGGLUTINATION	EFFECT ON RABBIT	REMARKS
3339	0	0		Survived†	Non-hemolytic non-coagulating strains (some weakly hemolytic strains have been included)
3342	0	0		Survived	
3341	0	0		D 6	
3319	0	0	1:160	D 8	
3330	++	0	0	D 8	
3320	++	0	0	Survived	
3316	++	0	0	Survived	
3231	++	0	0	Survived	
3230	++	0	0	Survived	
3227	++	0	0	Survived	
4807	++	0		D 7	
2834	0	0	1:640	Survived	
3163	0	0	1:640	Survived	
3167	0	0	1:640	Survived	
4585	0	0		D 6	
3328	0	0	0	Survived	
3322	0	0	0	Survived	
2854	0	0	0	Survived	
3151	0	0	0	Survived	
3159	0	0	0	Survived	
3154	0	0	0	Survived	
3139	0	0	0	Survived	
3142	0	0	0	Survived	
4820	++	0	1:320	Survived	
4821	0	0	1:320	Survived	
4657	0	0		D 5	
4660	0	0		D 8	
4719	0	0	0	Survived	
4744	0	0		D 5	
4791	0	0	0	Survived	
4813	0	0	1:640	D 7	
5038	0	0	1:160	Survived	
5025	0	0		Survived	
5027	0	0		Survived	
4996	0	0		Survived	
4994	0	0		Survived	
4992	0	0	0	Survived	
4990	0	0	1:320	Survived	
5012	0	0	0	Survived	
5039	0	0	0	Survived	
5022	0	0	0	D 6	

* Using human plasma.

† Survived 10 days.

D, died (number following symbol indicates number of days after inoculation).

agglutinability, the non-hemolytic, non-coagulating strains were usually non-pathogenic for rabbits (table 2). There was a uniform pathogenicity of the positively reacting aureus strains regardless of whether the toxin was a coagulase or a hemolysin (table 3).

TABLE 3

Results of injection of 5 billion live Staph. aureus intravenously into rabbits

STRAIN NUMBER	HEMOLYSIS	COAGULASE*	AGGLUTINATION	EFFECT ON RABBIT	REMARKS
3066	0	++++	1:1280	D 1	Coagulating non-hemolytic strains
2923	0	+++	1:160	D 5	
2815	0	+++	1:320	D 3	
3314	0	++++	0	D 2	
3275	0	++	1:640	D 1	
3329	0	++		D 1	
3333	0	++	0	D 6	
3248	0	++	1:640	D 2	
3327	0	++		D 7	
4810	0	++	1:640	D 1	
4795	0	++		D 1	
4767	0	++++		D 2	
4747	0	++	0	D 1	
4742	0	++	1:640	D 1	
4548	0	+	0	D 5	
4590	0	++	1:320	D 1	
4665	0	++		D 1	
4669	0	+++	1:160	D 1	
4672	0	+++	1:1280	D 2	
4695	0	++++	0	D 2	
4704	0	++++		D 2	
3070	++++	0	1:640	D 1	Hemolytic non-coagulating strains
3072	++++	0	1:640	D 2	
3246	++++	0		D 2	
3279	++++	0	1:640	D 1	
4777	++++	0	0	D 1	

* Human plasma.

D, died (number following symbol indicates number of days after inoculation).

From a summary of these reactions (table 4), it will be seen that none of the rabbits inoculated with hemolytic or coagulating aureus strains survived eight days and only 12 per cent of those inoculated with coagulating albus strains survived this period.

On the other hand, over 75 per cent of the rabbits inoculated with non-coagulating albus strains survived. This was true both of the hemolytic and the non-hemolytic albus strains. Carrying this analysis still further, the "survival time" of the rabbits is plotted in figure 4. The hemolytic aureus group was most pathogenic. There was only a slight difference in the pathogenicity of the coagulating aureus and the coagulating albus strains. The hemolytic albus group was only slightly more pathogenic than the non-hemolytic, non-coagulating albus group. In view of the observation of Burnet previously referred to, viz., that oxalated medium alters the hemolytic activity of staphylococci, we decided to investigate staphylococcal hemolysins with a view to attempting a differentiation of the non-toxic albus hemolysin from the toxic aureus hemolysin. It was considered possible that the use of oxalated blood for hemolysis tests had permitted hemolysis by strains whose mechanism of hemolysis differed from the factors responsible for the hemolytic activity of aureus strains. Two procedures suggested themselves: (1) The use of defibrinated blood, and (2) the use of heparin as an anticoagulant. Parallel observations of the three types of blood revealed no significant difference (table 6).

TABLE 4

Summary of animal inoculation experiments

ORGANISM	NUMBER OF STRAINS TESTED	COAGULASE	HEMOLYSIS	DEATHS IN LESS THAN 10 DAYS	
				Number	Per cent
Staph. albus (74 strains)...........	16	+	0	14	88
	17	0	+	4	23
	32	0	0	8	25
	9	0	0	2	22*
Staph. aureus (26 strains)..........	21	+	0	21	100
	5	0	+	5	100

* Agglutinating strains.

The presence of a coagulase, even in small amounts, was associated with toxicity for rabbits. There was no significant difference in the pathogenicity of strains giving a ++++ reaction (clotting plasma in one-half hour), those giving a +++ reaction (clotting plasma in one hour), those giving a ++ reaction (clotting plasma in two hours) and those giving a + reaction (clotting plasma in three hours). The time factor does not seem to be strictly constant and should be interpreted with caution (table 8).

TABLE 5

Comparison of hemolysis of staphylococci growing on blood agar prepared from different types of blood

LABORATORY NUMBER	TYPE	OXALATED BLOOD	DEFIBRINATED BLOOD	HEPARINIZED BLOOD	INCUBATION PERIOD
					hours
5216	Aureus	++++	++++	++++	48
5221	Aureus	+++	+++	+++	48
5224	Aureus	++	++	++	48
5225	Aureus	++	++	++	48
5228	Albus	0	0	0	48
5231	Albus	+	+	+	48
5204	Albus	+++	+++	+++	48
5197	Albus	++++	++++	++++	48
5198	Albus	++++	++++	++++	48
5233	Aureus	++++	++++	++++	48
5234	Albus	0	0	0	48
5235	Aureus	++++	++++	++++	48
5236	Albus	0	0	0	48
5237	Aureus	++++	++++	++++	48
5238	Albus	+	0	+	24
5239	Albus	++	0	+	24
5240	Aureus	+++	++++	+++	24

These findings stress the importance of separating staphylococcus strains on the basis of color, hemolysin and coagulase production. The agglutinin reaction will receive treatment in a later paper. The color determination is important because, if the strain is yellow, it is probably pathogenic, while, if it is white or creamy white, it is probably non-pathogenic. In certain cases it was noted that while the twenty-four-hour growths on proteose lactose agar appeared to be creamy white, the mass culture obtained by gathering the growth of an entire plate into a small area had a distinct yellow color. Many of these strains were quite toxic. This suggested that some of the anomolous reactions might have been due to errors in the determination of the color. Since the pigment had been shown to be a carotin, we attempted to modify the colors of surface growths by incorporating substances which might possibly contain mother substances for this class of pigments. Among the substances tried were corn meal, carrot infusion, carotene crystals and cod-liver oil. None of these were capable of enhancing the production of the yellow pigment in pale yellow strains. The color was slightly intensified on Loeffler's medium but the maximum color development was observed by incubating the strains for forty-eight hours instead of twenty-four hours, as suggested by many authors. By this technic, many of the supposed albus strains were found to be of the aureus type.

The determination of the coagulase is important because, regardless of its color, a coagulating strain is probably pathogenic. The determination of the hemolysin is important because, if the strain is yellow, it is probably pathogenic, while, if it is white and does not coagulate plasma, it is likely to be non-pathogenic. Consequently, we believe that these tests should be included in a study of the pathogenicity of staphylococci.

Both albus and aureus strains may be pathogenic for rabbits, but many of these pathogenic strains may not be agglutinable by the homologous serum. On the other hand, a few of the albus strains, which would have been considered of doubtful significance on the basis of the hemolysis and coagulase tests, were agglutinable. However, we were unable to demonstrate pathogenicity for these non-toxic agglutinable strains by the method used.

Since the Shwartzman phenomenon is considered a function of bacterial toxins, strains whose effect on rabbits was known were tested for their ability to elicit the phenomenon by the following technic:—A twenty-four-hour growth of the monophasic strain on proteose lactose agar (lactose agar in which Difco proteose peptone had been substituted for the usual peptone) was washed off with one per cent phenol in saline and the suspension was diluted to a concentration of 50 billions per cc. After testing for sterility, 0.1 cc. was injected subcutaneously into the depilated flank of a rabbit. The following day, 3 cc. was injected intravenously into the same animal and the site of the subcutaneous inoculation was watched for several hours.

TABLE 6

*The relationship of the Shwartsman phenomenon to other reactions of staphylococci**

ORGANISM	NUMBER	HEMOLYSIS	COAGULASE	AGGLUTINATION	RABBIT INOCULATION	SHWARTSMAN REACTION
Staph. albus.........	5025	0	0		D 9	0
Staph. albus.........	5027	0	0		0	0
Staph. albus.........	4996	0	0		0	0
Staph. albus.........	4994	0	0		0	0
Staph. albus.........	4992	0	0	0	0	0
Staph. albus.........	4990	0	0	1:320	0	0
Staph. albus.........	5012	0	0	0	0	0
Staph. albus.........	5039	0	0	0	0	0
Staph. albus.........	5038	0	0	1:160	D 9	2.0 x 2.0 cm.
Staph. albus.........	5022	0	0	0	D 6	0.7 x 0.6
Staph. albus.........	5061	0	+	0	0	2.0 x 1.5 (pustule)
Staph. albus.........	4980	0	++		0	0.5 x 0.5
Staph. albus.........	5138	+++	0	1:160	0	0.5 x 0.5 (papule)
Staph. albus.........	5135	+++	0	1:640	D 7	1.0 x 1.0 (papule)
Staph. albus.........	4964	+++	0	1:640	0	1.0 x 1.0 (macule)
Staph. aureus........	5181	0	+	1:10240	D 4	0
Staph. aureus........	5182	0	+	1:320	D 1	3.0 x 3.0 (papule)
Staph. aureus........	5183	0	+	1:640	D	1.5 x 2.0 (nodule)
Staph. aureus........	4844	0	+++	1:640	D 1	1.0 x 1.0 (pustule)

* These strains were not included in tables 1, 2 and 3 or fig. 4.

Preliminary tests had indicated that strains giving negative toxicity tests showed only a slight pinkish discoloration, while toxic strains produced markedly congested indurated areas which, in one instance, measured 3.0 x 3.0 cm. in diameter. Burnet (1931) obtained a negative reaction with a white staphylococcus. A more extensive series of tests was undertaken. Ten strains of *Staph. albus* giving negative toxicity tests were injected intravenously into rabbits to confirm the results of these tests. The same strains were then tested for their ability to elicit the Shwartzman phenomenon. A fresh rabbit was used for each test. Monophasic toxic strains were then subjected to similar experimentation. The following results were obtained (table 6).

Two of the non-toxic control strains produced reactions. They were weakly pathogenic when introduced intravenously. Another non-toxic control strain killed the rabbit in nine days but gave a negative Shwartzman reaction. The rest of the non-toxic strains reacted negatively to both tests. All toxic strains except one gave positive Shwartzman reactions although two of them failed to kill rabbits in ten days. Of interest are the reactions obtained with the hemolytic albus strains. The Shwartzman reaction was positive with all three although only one was pathogenic intravenously. These strains were also agglutinable but it is not likely that the agglutinogen was responsible for the Shwartzman reaction because an agglutinable non-toxic strain reacted negatively.

TABLE 7

Comparison of hemolysis and coagulase reactions of staphylococci

ORGANISM	NUMBER OF STRAINS EXAMINED	PER CENT OF STRAINS REACTING POSITIVELY	
		Hemolysis (++++ and +++)	Coagulase (++++, +++, ++ and +)
Staph. albus	1852	13.4	11.9
Staph. aureus	690	51.7	88.0

These observations give further support to the prevailing opinion that the Shwartzman phenomenon is activated by bacterial toxins and confirm Burnet's observation (1931) that the activity of a preparation as a Shwartzman reagent is apparently parallel to its killing power.

These results are of equal significance in strengthening the idea that albus strains are degenerate aureus strains. Most of the

TABLE 8

Comparison of coagulase tests on different occasions

DATE	PLASMA USED	STRAIN	RESULT
October 2	Rabbit no. 1	*Staph. aureus* no. 1	+++
7			++
12			++
21			++++
31			+++
November 1	Rabbit no. 2	*Staph. aureus* no. 1	+++
November 3	Rabbit no. 3	*Staph. aureus* no. 3	+++
8			++
11			+++
October 3	Human no. 1	*Staph. aureus* no. 1	+++
5	2		+++
7	2		+
	3		++
	4		++
12	5		+
	6		+
	7		+
15	8		+
	9		+
October 15	Human no. 10	*Staph. aureus* no. 2	+++
	11		+++
	12		++
	13		++
17	14		+++
	15		+++
18	16		++
19	17		+++
October 21	Human no. 18	*Staph. aureus* no. 3	++
	19		++
22	19		+
	20		++
	21		++
	22		++
	23		+
	24		+
	25		+
	26		+
	27		+

albus strains tested were lacking in pathogenic properties. The majority of the potentially toxic albus strains were less toxic than the aureus strains. The albus hemolysin is considerably less toxic for rabbits than the aureus hemolysin.

SUMMARY

Although most pathogenic types of staphylococci are hemolytic, and the hemolysis test is considered a reliable guide to the toxicity of the staphylococcus group, it is evident from the literature that certain pathogenic strains are non-hemolytic. In our series, only 51.7 per cent of 690 strains of *Staphylococcus aureus* were hemolytic.

In studying complementary tests for their correlation with pathogenicity of these non-hemolytic strains, the importance of the coagulase-activity test was confirmed. Of 690 strains of *Staphylococcus aureus* tested, 88 per cent coagulated oxalated plasma. When 1852 strains of *Staphylococcus albus* were similarly tested, only 11.9 per cent gave the coagulase reaction. Pathogenic non-hemolytic strains coagulated oxalated plasma.

Although aureus strains are usually more pathogenic than albus strains, the color, which should be determined after forty-eight hours' incubation, does not always furnish a reliable guide to the toxicity of a strain. Certain aureus strains are non-pathogenic, while a few albus strains are pathogenic for rabbits.

Strains reacting positively to either the hemolysis or coagulase test were injected into rabbits. Negatively-reacting strains were similarly injected to serve as controls. A few negatively-reacting but agglutinable strains were also tested. The following results were obtained: (a) 75 per cent of 32 strains reacting negatively to the hemolysis, coagulase and agglutination tests did not kill rabbits in ten days; (b) 78 per cent of nine agglutinable strains which reacted negatively to the hemolysis and coagulase tests did not kill rabbits in ten days; (c) 77 per cent of 17 hemolytic strains of *Staphylococcus albus* did not kill rabbits in ten days; (d) 88 per cent of 16 coagulating *Staphylococcus albus* strains killed rabbits in less than ten days; (e) all the hemolytic and the coagulating strains of *Staphylococcus aureus* killed rabbits in

less than ten days; (f) of the rabbits which died, death occurred in a shorter time with the aureus than with the albus strains.

There was a close parallel between the results of toxicity tests, as determined by hemolysis and coagulase activity tests, the pathogenicity of these strains when injected intravenously and their ability to produce the Shwartzman phenomenon.

There was no difference in the behaviour of strains when tested for hemolysis on rabbit blood agar which had been prepared from either oxalated, heparinized or defibrinated blood.

The simultaneous use of hemolysis and coagulase-activity tests and a careful determination of the color after forty-eight hours' incubation seems to afford a reliable combination of tests for the study of the pathogenicity of staphylococci. Omission of any of these tests may result in failure to recognize certain toxic strains.

CONCLUSIONS

1. A knowledge of the hemolytic activity of staphylococci is insufficient as a single serologic test of pathogenicity of this species.
2. A determination of coagulase activity is an important adjunct to the hemolysis test. Coagulating strains are usually pathogenic regardless of their hemolytic activity.
3. Coagulase-positive strains are usually pathogenic, regardless of the color produced on solid media.
4. On the other hand, the importance of the hemolysis test depends on an accurate determination of the color produced by the strain. Hemolytic non-coagulating albus strains are probably non-pathogenic but hemolytic aureus strains are usually pathogenic, regardless of their coagulase.
5. For the serologic recognition of toxic types of staphylococci, it is imperative to use at least the hemolysin and coagulase activity tests and a careful determination of the color. With a combination of these three reactions it is possible to estimate the toxicity of a strain with a high degree of precision.

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CULTIVATION OF ORGANISMS CONCERNED IN THE OXIDATION OF THIOSULFATE

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Although numerous investigations deal with the isolation and description of bacteria which decompose thiosulfate and other incompletely oxidized sulfur materials, considerable confusion still exists concerning certain aspects of the nutrition of these bacteria and the significance of the sulfur materials in their development. The present report includes studies on the influence of environmental conditions upon the development of some bacteria which up to this point at least would be classified as species of the genus *Thiobacillus*, Beij. The cultures used in these studies have been previously described in some detail (Starkey, 1934a). They may be briefly characterized as follows.

They are all Gram-negative, non-sporulating, small, rod-shaped bacteria. *Th. novellus* increases the acidity of mineral media containing thiosulfate and develops a uniform turbidity of the solution. This is a facultative autotrophic organism. It was described as a new species in the preceding report (Starkey, 1934a).

Culture B is closely related to the bacteria described by Trautwein (1921; 1924) and called *Th. trautweinii*, Bergey. It was fortunate that two of Trautwein's three cultures were available in this laboratory, having been supplied by their discoverer over ten years ago. Since that time they have been regularly transferred on nutrient agar. They show the same general characteristics originally ascribed to them. Their original identification as cultures T and K have been retained in this report. Culture B, isolated in this laboratory and T and K grow well on most of the common laboratory media but make little visible growth in the mineral medium containing thiosulfate. As a result of their development in this solution, thiosulfate disappears and the reaction becomes more alkaline.

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Culture C fails to grow on organic media in the absence of thiosulfate or sulfur. In the mineral medium, thiosulfate is decomposed with the precipitation of considerable sulfur, and the development of uniform turbidity throughout the solution. The reaction becomes increasingly more acid as growth progresses. This culture shows many features suggesting its identity with *Th. thioparus*, Beij. and is called by this name in the following pages.

INFLUENCE OF REACTION

The medium commonly used in these studies was prepared of the following materials and distributed in 100 cc. portions in 250 cc. Erlenmeyer flasks:

	gm.
Tap water	1,000
$MgSO_4\cdot 7H_2O$	0.1
$CaCl_2\cdot 2H_2O$	0.1
$MnSO_4\cdot 2H_2O$	0.02
$FeCl_3\cdot 6H_2O$	0.02
$(NH_4)_2SO_4$	0.1
$Na_2S_2O_3\cdot 5H_2O_4$	10.0
K_3PO_4, K_2HPO_4, or KH_2PO_4 or mixtures of these as stated for each experiment	2.0

The ammonium sulfate and sodium thiosulfate were sterilized separately. In this experiment, mixtures of phosphates were used to create a range of reactions from pH 9.0 to 4.8. Media at each reaction were inoculated with pure cultures of the five bacteria. Periodically the media were examined for extent of decomposition of thiosulfate and change in reaction. Residual thiosulfate was determined by titration with 0.01 N iodine solution. Reaction was measured colorimetrically.

Some of these results are shown in figure 1. In the upper portion of the figure is recorded the extent of decomposition of the thiosulfate and in the lower portion the pH numbers of the same cultures. It is apparent that culture B and Trautwein's cultures

T and K react qualitatively alike. Decomposition developed from pH 9.0 to 6.0 in all cases and also at pH 5.5 with cultures B and K. Maximum development took place in media which were initially acid—pH 5.5 or 6.0. In the early stages, maximum oxidation was found at pH 7.2 for B and 6.5 for K. The pH rose rapidly, reaching at least 9.8 with cultures T and K in two days in all cases where the initial reaction was above 6.5. No determinations were made covering reactions above 9.8. A slower increase is noted with culture B which made less growth at this period of two days. After the initial rapid increase, the reaction shows a tendency to decline as the cultures age. In some cases the reaction had dropped to that of the uninoculated controls at twenty-six days. The initial increase in pH is believed to be due to transformation effected by the bacteria; the subsequent drop is undoubtedly caused by secondary decomposition of the initial products as will be brought out in a later communication.

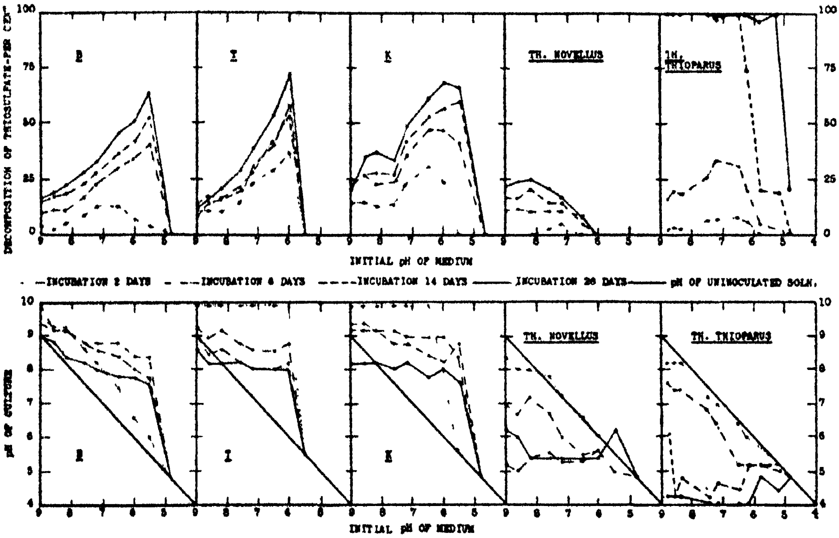

FIG. 1. DECOMPOSITION OF THIOSULFATE AND CHANGES IN REACTION BROUGHT ABOUT BY BACTERIA DEVELOPING IN MEDIA DIFFERING IN INITIAL REACTION

Trautwein (1921) noted practically the same decomposition of thiosulfate between pH 6.9 and 9.2 with best oxidation at pH 7.9 to 8.5. He stated that the culture solutions became alkaline during development of the bacteria except at high initial pH where the pH dropped in eight days.

Th. novellus is most active at alkaline reactions and makes slight development where the reaction is initially acid. Maximum oxidation occurred in media initially between pH 8.0 and 9.0. No growth was observed where the initial reaction was below pH 6.0. Even at the point of maximum development only about one-third as much thiosulfate was oxidized in twenty-six days as by cultures B, T or K. However, the change in reaction was extreme, but in the opposite direction from the preceding cultures. The acidity gradually increased to about pH 5.0 to 5.5 which seemed to be unfavorable for further growth. The halophilic bacterium of Saslawsky (1927) and Saslawsky and Harzstein (1930) oxidized thiosulfate completely to sulfate rendering the medium acid. In some respects this organism behaves similarly to *Th. novellus*. Guittonneau's culture also oxidized thiosulfate to sulfate (1925; 1927; 1928).

Th. thioparus initially grew best in media adjusted close to neutrality but, after twenty-six days, decomposed practically all of the thiosulfate in media initially having reactions from pH 5.2 to 8.8. The rapid increase in acidity delayed development in the solutions which were originally acid. This is much the most active of the cultures under investigation (excepting *Th. thiooxidans*) in several respects: rapidity of growth, wide range of pH over which development occurs, extreme acidity produced by decomposition of thiosulfate. The acidity increased rapidly to between pH 4.0 and 5.0. The medium of initial pH 6.5 changed to pH 3.4; this is shown only as pH 4.0 on the graph.

The medium supporting growth of the culture studied by Nathansohn (1902) remained alkaline and did not change in reaction in ten days. Since polythionates were detected in the solutions, it is possible he was not using a pure culture. Issatschenko and Salimowskaja (1929) and Lange-Posdeeva (1930) worked with cultures showing characteristics similar to those of *Th. thioparus*; they state that the reaction becomes acid during growth. Products of oxidation similar to these produced by *Th. thioparus* were formed by a culture studied by Salimowskaja (1931) but the pH of the medium increased during growth, as from an initial pH of 4.4 to pH 7.6.

In contrast with these cultures, *Th. thiooxidans* develops best in strongly acid media growing most rapidly between pH 2.0 and 3.0 but developing from below pH 1.0 up to 6.5 or 7.0 (Waksman and Starkey, 1923; Starkey, 1925). There appears to be little available information on reaction changes brought about by *Th. denitrificans* but, from the transformation ascribed to it, there is likelihood of an increase in acidity (Lieske, 1912; Tjulpanova-Mossevitch, 1930).

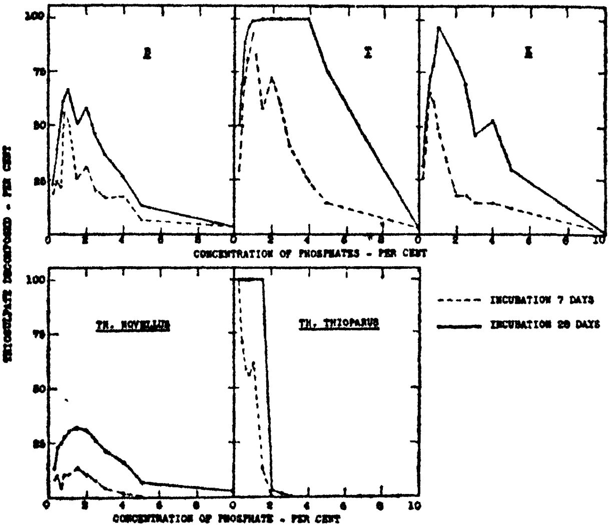

FIG. 2. INFLUENCE OF PHOSPHATE CONCENTRATION UPON DECOMPOSITION OF THIOSULFATE BY VARIOUS BACTERIA

CONTROL OF CHANGE IN REACTION

Media were prepared the same as for the preceding experiment with the exception of the phosphates. Since *Th. novellus* and *Th. thioparus* developed better in alkaline media, K_2HPO_4 was the only phosphate used, creating a pH of 7.8 irrespective of the phosphate concentration. For cultures B, T and K equal portions of K_2HPO_4 and KH_2PO_4 were used in the media buffering the solutions at pH 6.6. The phosphates were used in various concentrations from 0.2 to 10.0 per cent. The extent of decomposition of thiosulfate by the various cultures is shown in figure 2.

With cultures B, T and K there was an increase in pH; with *Th. novellus* and *Th. thioparus* the pH dropped. At the low phosphate concentration, inhibitive reactions were quickly reached; at high phosphate concentrations, the influence of the salt concentration was the factor determining activity. The curves are probably resultants of the effects of these two factors.

Buffering the media quite strongly to prevent extreme changes in reaction favored development in all cases. The concentration of phosphate salt commonly used in the media for many of the early experiments in these investigations was 0.2 per cent. It is apparent that this is not the optimum concentration for any of the cultures except *Th. thioparus* and even this organism decomposed all of the thiosulfate in 28 days in concentrations up to 1.5 per cent. *Th. thioparus* was least tolerant to high phosphate concentrations making very limited or no growth above 1.5 per cent. Concentrations of 1 to 2 per cent gave best results with *Th. novellus* but oxidation was quite meagre compared to the optimum development of the other organisms. Concentrations of 0.8 to 2 per cent seemed most favorable for cultures B, T and K and oxidation continued even at 5 per cent. Culture T was particularly active over a wide range of concentrations.

Another experiment was conducted in which the reactions of the media were periodically adjusted. The medium was the same as that initially described but contained 0.2 per cent K_2HPO_4 creating a reaction of pH 7.8. Part of the cultures were not altered during incubation. Other solutions were neutralized after the determinations for residual thiosulfate on the fifteen- and twenty-six-day periods using determined amounts of sterile 0.1 N NaOH for *Th. novellus* and 0.1 N H_3PO_4 for cultures B, T and K. Table 1 indicates that oxidation was favored in all cases by neutralization of the media. The products of oxidation of thiosulfate which are most inhibitive to further development of the organisms appear to be the OH-ions for cultures B, T and K and the H-ions for *Th. novellus*.

When bacterium B, T or K is cultivated with *Th. novellus* in a medium having a reaction close to neutrality, oxidation of thiosulfate is more extensive than when any of these organisms is cultured alone. This is probably due to the fact that *Th. novellus* tends to increase the acidity of the medium and B, T and K to decrease it during development. Since *Th. thioparus* grows very rapidly, over a wide range of reaction, oxidation of thiosulfate is not appreciably modified by the presence of any of the other cultures. The same applies to *Th. thiooxidans* except in special cases. When the initial reaction is close to the critical maximum pH (between 6.5 to 7.0), cultures B, T or K in association tend to make the reaction alkaline before growth of *Th. thiooxidans* becomes established. In some cases of such associations, growth of *Th. thiooxidans* is prevented in media that would be suitable

TABLE 1

Decomposition of thiosulfate by cultures in media periodically neutralized

CULTURE	NEUTRALIZED (+) OR UNNEUTRALIZED (−)	THIOSULFATE DECOMPOSED		
		15 days	26 days	39 days
		per cent	*per cent*	*per cent*
B_1	−	10.9	18.1	23.1
	+	10.9	19.8	29.6
B_2	−	8.2	9.7	13.0
	+	8.8	13.5	22.7
T_1	−	20.3	30.6	37.6
	+	22.1	37.1	52.0
T_2	−	29.5	48.5	58.9
	+	28.4	52.4	67.3
K_1	−	23.1	35.5	43.9
	+	23.7	41.5	57.1
K_2	−	22.8	35.3	42.9
	+	24.9	46.1	61.5
Th. novellus (1)	−	4.0	7.0	8.7
	+	3.8	13.8	24.8
Th. novellus (2)	−	10.2	12.3	13.2
	+	13.5	29.1	41.3

TABLE 2

*Composition of media used for culturing sulfur bacteria**

Medium Constituents	No. 1	No. 2	No. 3	No. 4	Nathansohn† (1902)	Beijerinck† (1903 a and b)	Beijerinck‡ (1904 a and b)	Jacobsen† (1912; 1914)	Lieske‡ (1912)	Trautwein§ (1921)	Salimovskaja‖ (1930)
$Na_2S_2O_3 \cdot 5H_2O$	10.0	10.0	10.0	10.0	5.0	5.0	5.0	5.0	5.0	2.0	10.0
K_2HPO_4	2.0	4.0	6.0	2.0		0.2	0.2	0.5	0.2		3.0
KH_2PO_4		4.0									
$Na_2HPO_4 \cdot 12H_2O$					5.0					0.2	
$CaCl_2$	0.1	0.1	0.1	0.1					Trace		0.2
$MgSO_4 \cdot 7H_2O$	0.1	0.1	0.1	0.1							
$MgCl_2 \cdot 6H_2O$					2.5	2.5	0.1	0.2	0.1	0.1	0.5
$(NH_4)_2SO_4$	0.1	0.1	0.1	0.1							
NH_4Cl						0.1		0.5		0.1	2.0
KNO_3					0.1		0.5		5.0		
$CaCO_3$ (or $MgCO_3$)				In excess (5.0)	In excess (5.0)		In excess (20.0)	In excess (20.0)			
Na_2CO_3							0.1				
$NaHCO_3$						1.0			1.0	1.0	
$FeCl_3 \cdot 6H_2O$	0.02	0.02	0.02	0.02				Trace	Trace		
$MnSO_4 \cdot 2H_2O$	0.02	0.02	0.02	0.02							

* On basis of grams per 1,000 cc. Distilled water was used for all except Nos. 1, 2, 3, 4, in which tap water was used. These four are original media.

† For *Th. thioparus.*

‡ For *Th. denitrificans.*

§ For *Th. trautweinii.*

‖ For an organism similar to *Th. thioparus.*

for development of the pure culture. On the other hand, *Th. novellus* or *Th. thioparus* may favor growth of *Th. thiooxidans* if they occur together in media that, initially, are slightly too alkaline for the last organism. This is due to the fact that both *Th. novellus* and *Th. thioparus* increase the acidity of the medium during growth. It was noted by Waksman and associates that sulfur and thiosulfate are oxidized more rapidly in solutions and soils by mixed cultures of sulfur bacteria than by the separated pure cultures (Waksman, 1922 a and b; Waksman, Wark, Joffe and Starkey, 1923).

DEVELOPMENT UPON VARIOUS MINERAL MEDIA

In table 2 is given a list of the media used in this study; some of these have been previously recommended for culturing members of the genus *Thiobacillus*. Numbers 1, 2, 3 and 4 are first described in this report. The calcium carbonate, ammonium sulfate and thiosulfate were sterilized separately in the autoclave. The sodium carbonate and bicarbonate were sterilized by passage through a Seitz filter. Almost all of the organisms developed in the media.

The media most suitable for development of each culture, judging from oxidation of thiosulfate at earlier periods and at the twenty-six day period are underlined in table 3. For cultures B, T and K, medium 2 seemed the best although Nathansohn's solution was quite suitable. *Th. novellus* made the best growth on Jacobsen's medium although medium 3 which was prepared especially for its cultivation was nearly as satisfactory at the twenty-six-day period; at earlier periods, medium 3 was superior to the others. *Th. thioparus* makes remarkably fine growth on a great variety of media. Observations at an earlier period (six days) indicated that medium 2 and the one of Trautwein were particularly favorable for rapid development.

INFLUENCE OF SOME MEDIUM CONSTITUENTS

Cultures B, T, K and *Th. novellus* became relatively inactive after continuous cultivation in thiosulfate solution. They were cultivated in sterilized soil to which sodium thiosulfate had been added but development in soil failed to alter their activity significantly.

With the expectation that some substances not commonly incorporated in the medium might favorably affect development of

TABLE 3

Decomposition of thiosulfate in various mineral media

MEDIUM	CONTROL	B	T	K	TH. NOVELLUS	TH. THIOPARUS
Thiosulfate decomposed, per cent*						
No. 1		4.5	23.4	22.5	13.4	100.0
No. 2		14.9	68.5	72.5	15.0	100.0
No. 3		2.5	22.2	21.7	31.0	100.0
No. 4		2.0	13.2	23.9	26.1	100.0
Nathansohn		12.9	56.9	52.6	20.0	31.9
Beijerinck†		0	4.1	9.5	0	2.3
Beijerinck‡		9.1	29.3	35.8	12.9	100.0
Jacobsen		6.3	41.4	40.5	36.3	100.0
Lieske		0	3.5	20.4	0	100.0
Trautwein		0	5.7	49.4	0	100.0
Salimowskaja		8.4	31.7	43.7	21.0	100.0
Reaction (pH)						
No. 1	7.8	8.0	8.8	8.6	5.4	4.4
No. 2	6.6	7.0	7.8	7.8	5.8	4.8
No. 3	8.0	8.0	8.8	8.6	6.4	6.8
No. 4	>9.8	>9.8	>9.8	>9.8	8.6	7.8
Nathansohn	7.4	7.6	8.2	8.0	7.0	7.2
Beijerinck†	>9.8	>9.8	>9.8	>9.8	>9.8	>9.8
Beijerinck‡	8.8	8.8	9.2	9.0	8.2	8.0
Jacobsen	8.4	8.6	8.4	8.4	8.0	8.0
Lieske	>9.8	>9.8	>9.8	>9.8	>9.8	6.0
Trautwein	9.6	>9.8	>9.8	>9.8	>9.8	4.0
Salimowskaja	7.4	7.6	8.0	8.2	5.8	4.4

* Incubation period 26 days.

† For *Th. thioparus.*

‡ For *Th. denitrificans.*

the cultures, a series of media were prepared containing, in addition to the constituents of medium 1 in table 2, bicarbonate (0.1 per cent), a small amount of an iron lignoprotein complex prepared by Waksman and Iyer (1932 a and b) or $NaNO_3$ (0.1 per cent). None of these substances alone or in combination increased growth significantly. In an additional experiment, certain organic substances were utilized (table 4). The yeast infusion was prepared according to the directions of Fred and Waksman (1928).

It is apparent from table 4 that organic substances greatly favor oxidation of thiosulfate by cultures B, T and K and exert no significant favorable or depressing effect on *Th. novellus.* However, growth of *Th. novellus,* as judged from the turbidity of the culture, was far more extensive on media 2–4 than on 1, but apparently decomposition of thiosulfate bears no close relationship to amount of growth in the presence of organic materials. Cultures B, T and K also made extensive growth in the organic media and decomposition of thiosulfate also increased, but probably not in proportion to the increase in abundance of cells.

TABLE 4

Influence of some organic materials upon decomposition of thiosulfate

MEDIUM NUMBER	SUBSTANCE ADDED	THIOSULFATE DECOMPOSED*			
		Culture B	Culture T	Culture K	Th. novellus
		per cent	*per cent*	*per cent*	*per cent*
1†		5.9	14.6	14.7	4.1
2	No. 1 + 0.01 per cent asparagine	20.9	27.8	28.6	5.6
3	No. 1 + 0 01 per cent peptone	12.7	26.0	29.3	3.9
4	No. 1 + 5 per cent yeast infusion	24.0	33.3	47.5	4.2

* Incubation period, eleven days.

† Same as medium 1, table 2.

INFLUENCE OF ORGANIC SUBSTANCES

In view of the apparent favorable effect of the organic substances on oxidation of thiosulfate by some of the cultures, a more extensive experiment was performed making use of the five different cultures and both carbohydrates and nitrogenous compounds. Three groups of media were prepared: (1) The first group included medium 1 (table 2) for *Th. thipoarus,* medium 2 (table 2) for cultures B, T and K, and medium 3 (table 2) for

Th. novellus. (2) This group received, in addition to the substances in group one, certain organic compounds in concentrations of 0.01, 0.1 or 0.5 per cent as indicated in figure 3. (3) Media of group three received no thiosulfate but in other respects were identical with the media of group 2. The organic materials, thiosulfate and ammonium sulfate were sterilized separately.

In figure 3 the white columns indicate the amount of thiosulfate decomposed in five days, the black columns show the change in twenty-two days. *Th. thioparus* made no growth in any of the solutions lacking thiosulfate as would be expected with a strict autotroph. The presence of organic materials appeared to exert no influence upon decomposition of thiosulfate. All of the thiosulfate had disappeared in all cases at twenty-two days as shown by the figure. Other determinations not recorded indicated that complete decomposition had been accomplished in all cases at thirteen days. It would thus appear that this organism is quite tolerant to appreciable concentrations of organic materials without being affected by them. Similar results were obtained for

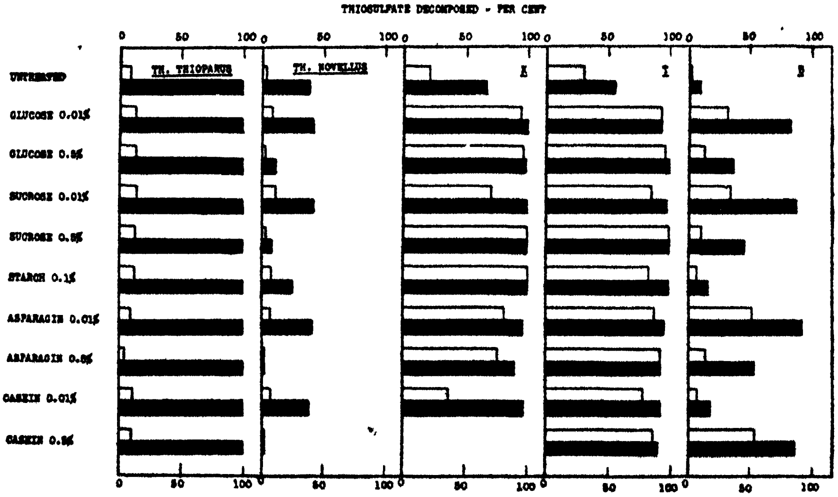

FIG. 3. DECOMPOSITION OF THIOSULFATE BY VARIOUS BACTERIA DEVELOPING IN MEDIA CONTAINING SOME ORGANIC MATERIALS

Incubation period five days for unshaded columns; twenty-two days for black columns pure cultures of *Nitrosomonas monocella* and *Nitrobacter agilis* by Nelson (1931), and for *Th. thiooxidans* by Waksman and Starkey (1923) and Starkey (1925).

In the initial studies with *Th. thioparus,* Nathansohn found that organic materials were not utilized, and that they had no injurious effects on the organism (1902). Beijerinck stated that no other source of carbon could replace carbonic acid (1904 a and b). Klein and associates (1923; 1926) presumed that they were using a similar organism but it developed upon organic substances. The culture of Salimowskaja grew in nutrient solution in the presence of thiosulfate (1931). The bacteria of Issatschenko and Salimowskaja (1928; 1929) were believed to benefit from organic matter since they appeared to develop better with nitrogen supplied as peptone or asparagine than in the ammonium or nitrate form.

Referring again to figure 3, it is apparent that *Th. novellus* did not decompose thiosulfate to any greater extent in the presence of the organic materials than in their absence. In fact, at the 0.5 per cent concentrations of the substances, oxidation was almost completely inhibited. This is particularly interesting in view of the fact that growth was much more extensive at the higher concentrations of organic materials and was always greater in the presence of the lower amounts of organic matter than in the strictly mineral medium. Growth appeared to be equally extensive in the media of series 3, which contained organic substances but lacked thiosulfate, as in the comparable media of series 2. In dealing with this culture which is a facultative autotroph it is interesting to note that, in the presence of two available different sources of energy, one may support extensive development while the other is attacked less rapidly than where it occurs alone. This recalls the frequently noted sparing effects of carbohydrates upon the decomposition of proteins or protein derivatives when both are supplied in the same solution to certain bacteria (Waksman, 1932). It seems probable that in view of the fact that much more extensive growth occurs on organic than on thiosulfate media, the organic materials are metabolized more readily, and, in the presence of both substances, support the development of practically all of the cells. The culture of Guittonneau (1925) also grew upon both thiosulfate and organic compounds.

Decomposition of thiosulfate by cultures B, T and K was greatly increased in the media containing organic substances (fig. 3). Both T and K made extensive growth in the media containing organic matter both where thiosulfate was present and where none was added. Heavier growth was apparent in the cultures containing the higher concentrations of organic matter. Culture B reacted similarly except that there was practically no growth in the presence of starch or 0.1 per cent casein. Although oxidation of thiosulfate is greater where the bacterial cells are more numerous in the media with organic matter, there is not an absolute correlation between growth and oxidation since more growth of culture B occurred in the media containing the higher concentrations of glucose, sucrose and asparagine. However, there was less decomposition of thiosulfate at the higher concentration. Trautwein was also able to cultivate his cultures on a great variety of organic media (1921; 1924).

On the basis of their reactions to organic materials, the organisms thus fall into 3 groups: (1) The strict autotroph, *Th. thioparus*, (2) the facultative autotroph, *Th. novellus*, (3) a special group of organisms represented by cultures B, T and K.

In regard to the tolerance of *Th. thioparus* for organic substances it may be of interest to note that this bacterium persisted for at least ten days on nutrient agar which contains little or no apparent energy-yielding substance to the cells; no growth was apparent during this period but typical development occurred upon inoculation into the thiosulfate medium.

The continuous cultivation of *Th. novellus* and bacteria B, T and K upon nutrient agar as compared with culture upon thiosulfate agar, for a considerable period of time, does not appreciably alter their behavior. Slants of these cultures were compared by inoculation into the mineral thiosulfate medium after monthly transfer upon the agar media for about one year. The differences were slightly in favor of the cultures grown upon nutrient agar, but this can be explained by the fact that greater numbers of cells were inoculated from the nutrient agar slants since growth was more extensive upon this medium.

Trautwein reported that his cultures did not lose their capacity to attack thiosulfate by growth for 9 months on nutrient agar (1921). Lange-Posdeeva was able to carry an organism, similar to *Th. thioparus*, on thiosulfate agar for three years without its losing the capacity to decompose thiosulfate in solution media (1930). The purple sulfur bacteria, although facultatively autotrophic, do not readily lose their autotrophic habit. Van Niel found that, after transferring them for four months, every three weeks, on both Na_2S and yeast extract media, the cultures from the organic medium developed as well as the Na_2S strains when again transferred to the Na_2S medium (1931). During his early studies with *Th. denitrificans*, Beijerinck believed that two organisms existed in his denitrifying solutions, one heterotrophic, the other autotrophic (1904 a and b). At a later date (1920) he was convinced that the two organisms were originally alike but that the autotrophic function of *Th. denitrificans* becomes permanently lost by development upon organic media. Similar statements were made by him about the nitrifying bacteria (1914). The criticisms of Winogradsky (1922) and Bonazzi (1923) concerning the stability of the autotrophic characteristic of the nitrifying bacteria seem equally applicable to the sulfur bacteria. The available evidence justifies the conclusion that the autotrophic function is very stable and not readily lost; the bacteria retain their individual characteristics in the presence of organic matter and after exposure to a great variety of other cultural environments.

AVAILABILITY OF VARIOUS SOURCES OF NITROGEN

In this experiment the media used were the same as numbers 1, 2, and 3 in table 2 except for the nitrogen source, with No. 1 for *Th. thioparus*, No. 2 for cultures B, T, K, *Ps. fluorescens* and *Achrom. hartlebii*, and No. 3 for *Th. novellus*. The amounts of the various nitrogenous substances used are indicated in figure 4. It was discovered during experiments reported elsewhere (Starkey, 1934 b) that *Ps. fluorescens* and *Achrom. stutzeri* attack thiosulfate in much the same manner as cultures B, T and K, which explains their use in this study.

After twenty-six days, the culture solutions were tested to determine the amounts of thiosulfate lost by decomposition, for changes in pH and ammonia content and for nitrite. It is evident that *Th. novellus* decomposed relatively small quantities of thiosulfate in any of the media, not oxidizing over 17 per cent in any case. Practically equal oxidation occurred in media to which no nitrogen was added as in the presence of the nitrogenous materials, suggesting that in most cases, nitrogen was probably not the factor limiting activity. Growth was greatly favored in all of the media receiving the organic substances although oxidation of thiosulfate was not increased. Since growth and ammonia production were favored by the organic materials these substances are undoubtedly available sources of nitrogen. Nitrate was not reduced to nitrite by this culture.

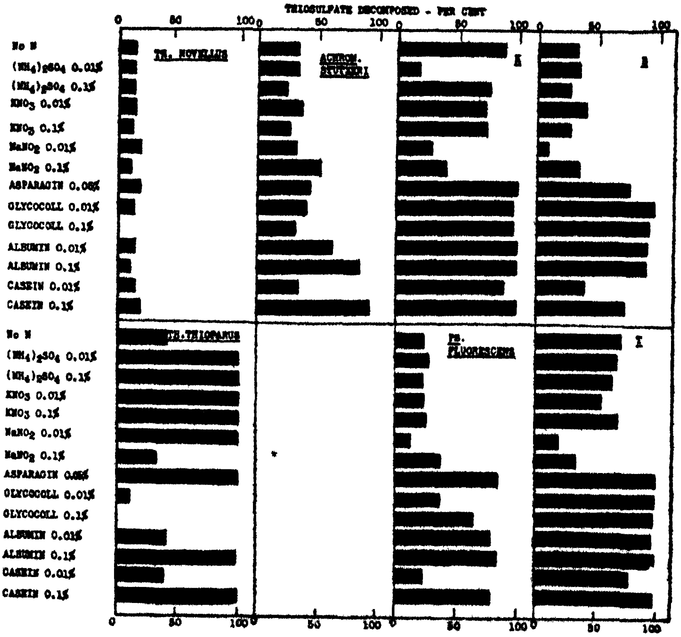

FIG. 4. DECOMPOSITION OF THIOSULFATE BY BACTERIA IN MEDIA CONTAINING VARIOUS NITROGENOUS SUBSTANCES

Th. thioparus showed marked response to the various nitrogenous materials and decomposed all of the thiosulfate in the media which were favorable to growth. All three inorganic sources of nitrogen, ammonia, nitrate and nitrite can be utilized, but the last is toxic in concentration of 0.1 per cent. Nitrate was reduced with the accumulation of considerable nitrite. Glycocoll was quite toxic and there was practically no oxidation of thiosulfate nor evidence of growth at either concentration. The data in the figure suggest that *Th. thioparus* utilized nitrogen from asparagine, albumin and casein. This was unexpected in view of the fact that in none of the previous observations has there been any suggestion that this bacterium could attack organic compounds. It is still believed that the organic nitrogenous materials were not decomposed. No ammonia was formed in the media containing the asparagine, albumin or casein which could be said to be due to bacterial attack and there was no evidence that growth of the culture was greater in the presence of the organic materials than of the inorganic compounds. With the low concentrations of albumin and casein, oxidation of thiosulfate was practically the same as in the absence of nitrogen although the amount of nitrogen present should have been quite ample to supply the need of the bacterium if it had been available. It is believed that at the higher concentrations, 0.05 per cent asparagine and 0.1 per cent albumin and casein, sufficient ammonia was split from the organic compounds during sterilization under steam pressure to satisfy the requirements of growth for complete decomposition of thiosulfate. Ammonia was recovered in small amounts from the controls after sterilization. The conclusions of Issatschenko and Salimowskaja (1928; 1929) that an organism similar to *Th. thioparus* was able to utilize nitrogen in organic combination from peptone and asparagine as well as from NH_4^+ or NO_3^- may also need qualification for a similar reason. *Th. thiooxidans* requires ammoniacal nitrogen and cannot use nitrate which is toxic (Starkey, 1925).

Nitrite appears to be somewhat toxic to culture B, and more so to T and K. It is not believed that the development of cultures B, T, K, *Ps. fluorescens* and *Achrom. stutzeri* indicate utilization or lack of availability of the inorganic compounds since actual *growth* was very limited in all cases. Even nitrite was not toxic for *Ps. fluorescens* or *Achrom. stutzeri.* In the presence of organic materials, oxidation of thiosulfate was increased in practically all cases where there was evidence of increased growth of the organisms. Ammonia was formed from glycocoll by cultures B, T, K and *Ps. fluorescens* and from albumin and casein by *Achrom. stutzeri*. Nitrate was reduced to nitrite by culture K. All of these cultures grew very well upon asparagine and glycocoll.

TABLE 5

Oxidation of elemental sulfur

CULTURE	SULFATE-S IN 100 CC. MGM.		TOTAL S IN 100 CC. MGM.	
	14 days	34 days	14 days	34 days
		Medium 1		
Control	2.9	2.4	3.2	2.0
B	2.9	2.3	4.0	2.3
T	2.9	2.3	3.2	2.9
K		2.9	3.2	2.6
Th. novellus	4.0	2.9	3.4	2.6
Th. thioparus	13.2	17.2	11.2	17.5
		Medium 2		
Control	4.3	3.0	4.9	4.3
B	4.3	3.2	4.3	4.6
T	4.4	3.2	4.0	4.9
K	4.3	3.2	4.6	4.3
Th. novellus	4.6	4.0	3.4	4.6
Th. thioparus	29.3	42.9	29.5	39.5

OXIDATION OF SOME INORGANIC SULFUR MATERIALS

The two media prepared for these studies had the same composition as the medium initially described in this report with the following exceptions. Elemental sulfur (2 per cent) was substituted for thiosulfate. Medium 1 received 7.5 grams K_2HPO_4 per liter. Medium 2 received 2 grams KH_2PO_4 and 2 grams $CaCO_3$ per liter. As indicated in table 5, *Th. thioparus* was the only culture which oxidized sulfur in the mineral media, development being much more active in the presence of $CaCO_3$. Sulfate was the only product of oxidation detected. However, the oxidation of sulfur by this bacterium is very slow compared to its oxidation of thiosulfate. No sulfate or other product of oxidation was found in the media inoculated with the other bacteria.

In other experiments, these cultures were inoculated into media containing sodium dithionate (0.05 and 0.5 per cent), sodium sulfite (0.05 and 0.5 per cent) and sodium sulfide (0.05 per cent). All of the sulfite became oxidized to sulfate in a few days in all of the inoculated and uninoculated media and none of the change could be ascribed to bacterial action. Sulfite is so unstable in contact with air that it appears unlikely that organisms of the type under consideration could utilize it as a source of energy. None of the cultures seemed able to attack sulfide in the concentration used. Dithionate appeared to be slowly oxidized by *Th. thioparus* but not by the other cultures. This oxidation was accompanied by the appearance of sulfate and an increase in acidity.

The results of studies by various investigators using several different organisms indicate that all of the sulfur bacteria of the genus *Thiobacillus* are able to utilize thiosulfate. The green sulfur bacteria utilize only sulfide, but the purple bacteria use thiosulfate, sulfite, elemental sulfur and sulfide (Van Niel, 1931; Van Niel and Muller, 1931). Previous investigators are also in general agreement that thiosulfate is more readily oxidized than other sulfur materials (Nathansohn, 1902; Beijerinck, 1904a and b; Lange-Posdeeva, 1930; Lieske, 1912; Gehring, 1915; Guittonneau, 1928; Salimowskaja, 1931). This does not appear to be the case with *Th. thiooxidans* which rapidly oxidizes both sulfur and thiosulfate (Waksman and Starkey, 1923; Starkey, 1925a and b). The culture of Rountree reacts similarly (1933) but cannot develop under the extreme acidity tolerated by *Th. thiooxidans.* In several reports it is stated that organisms resembling *Th. thioparus* are able to oxidize sulfur (Issatschenko and Salimowskaja, 1928 and 1929; Lange-Posdeeva, 1930; Saslawsky, 1927). The most significant results are those of Jacobsen, (1912) who observed that the bacterium could also oxidize sulfide (1914) although it was very sensitive to this substance (see also Nathansohn, 1902; Beijerinck, 1904a and b). *Th. denitrificans* oxidizes sulfur (Beijerinck, 1904a and b; Lieske, 1912; Gehring, 1915; Tjulpanova-

Mossevitch, 1930) and was found to make poor or fair development upon sulfide, sulfite (Beijerinck, 1904a) and dithionate (Lieske, 1912; Gehring, 1915) Salimowskaja's culture grew only on thiosulfate (1931). Trautwein claimed that sulfide and elemental sulfur were oxidized by his bacteria (1921). Due to inadequate control in many of these studies, the actual diversity of sulfur materials suitable as sources of energy for some of the species of *Thiobacillus* still remains uncertain.

SUMMARY

Results are reported of studies concerned with the cultivation of several bacteria generally classified as members of the genus *Thiobacillus*, Beij. These bacteria include an organism which is strictly autotrophic and appears to be much the same as *Th. thioparus*, a facultative autotrophic bacterium *Th. novellus* recently described as a new species, and 3 cultures (B, T, K) differing from these. The first two bacteria increase the acidity during growth in thiosulfate media; cultures B, T and K decrease the acidity. Development of *Th. thioparus* and *Th. novellus* is favored in media which are initially alkaline in reaction; B, T and K develop best in media initially slightly acid and well buffered. Buffering the media strongly, also favored oxidation of thiosulfate by *Th. novellus* but did not appreciably affect *Th. thioparus*. Similar favorable effects on oxidation resulted from periodically adjusting the reaction of solutions supporting development of the cultures. Associations of pairs of cultures, one of which increases the acidity and the other decreases the acidity, frequently effect more rapid oxidation of thiosulfate than the pure cultures alone.

Various mineral media previously suggested for study of species of *Thiobacillus* were used with the pure cultures. The media devised by the author were, in most cases best suited for the cultures but many media were favorable for *Th. thioparus*. Several substances added to the solution media failed to favor oxidation of thiosulfate by *Th. thioparus* and *Th. novellus*. Organic materials greatly increased both growth and decomposition of thiosulfate by B, T and K. Although growth of *Th.*

novellus increased in the presence of organic materials, the decomposition of thiosulfate was not increased and was frequently decreased. Oxidation of thiosulfate by *Th. thioparus* did not increase in the presence of organic materials and this culture failed to grow on organic substances. *Th. thioparus* was not affected by as much as 0.1 or 0.5 per cent concentration of some organic materials. Continuous cultivation of *Th. novellus* and cultures B, T and K on nutrient agar for a year did not alter their ability to attack thiosulfate when they were again introduced to the solution medium.

Th. novellus and cultures B, T and K use a variety of organic materials as sources of nitrogen. *Th. thioparus* probably is able to use only inorganic nitrogen; nitrite, nitrate and ammoniacal nitrogen are used. Nitrate is reduced to nitrite.

Of the various sources of sulfur used, thiosulfate is the only one which is rapidly oxidized. Elemental sulfur is slowly oxidized to sulfate by *Th. thioparus* but not by B, T, K or *Th. novellus*. Sulfite is oxidized so rapidly by chemical action that it seems unlikely that these bacteria are concerned in its oxidation. No oxidation of sulfide by the bacteria was observed. Dithionate appeared to be slowly oxidized to sulfate by *Th. thioparus*.

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THE PRODUCTION OF POLYTHIONATES FROM THIOSULFATE BY MICROÖRGANISMS

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In recent reports the author has discussed the isolation, characteristics and cultivation of some bacteria which are able to oxidize thiosulfate (1934a and b). The cultures include: (1) *Th. novellus*, a facultative autotrophic bacterium, which oxidizes thiosulfate to products which increase the acidity of the medium; (2) a strict autotroph apparently *Th. thioparus*, which oxidizes thiosulfate with copious precipitation of elemental sulfur and formation of acid (Bergey et al., 1930); culture B which reacts qualitatively the same as Trautwein's bacteria T and K (1921; 1924), which were also available for these studies (see *Th. Trautweinii* Bergey (1930)). They oxidize thiosulfate with very little apparent growth but bring about a decrease in the acidity of the medium from the products formed. They grow luxuriantly upon most of the common laboratory media. A strict autotroph, *Th. thiooxidans*, also used in these studies was the original strain isolated by Waksman and Joffe (1921). This bacterium oxidizes thiosulfate to sulfate (Waksman and Starkey, 1923). Trautwein assumed that the incompletely oxidized sulfur compounds detected in his culture solutions were dithionate and tetrathionate (1921). The formation of such compounds could account for the reaction changes observed for cultures B, T and K.

The qualitative test for polythionates applied in the present studies depends upon the well established reactions of polythionates in alkali solutions. According to Kurtenacker and Goldbach (1927) and Guittonneau and Keilling (1932b) the polythionates are decomposed by alkalies as follows:

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	SULFITE-S	THIOSULFATE-S
	per cent	*per cent*
Trithionate	66	33
Tetrathionate	25	75
Pentathionate	0	100

Since the polythionates do not react with iodine and, since both sulfite and thiosulfate are oxidized by iodine, the presence of polythionates can be detected by the differences in iodine titrations before and after the alkali treatment. The test was conducted as follows. Five cubic centimeters of the solution, filtered free of elemental sulfur, were titrated with 0.01 N iodine solution to obtain a measurement of the amount of undecomposed thiosulfate. A similar 5 cc. aliquot was added with 5 cc. of 10 per cent KOH with a small amount of distilled water, thoroughly mixed, and heated in a boiling water bath for five minutes. After cooling, 10 cc. of 10 per cent acetic acid were added and the solution was titrated with 0.01 N iodine solution. An increase in titration over that initially performed for residual thiosulfate indicates the presence of polythionates.

The mineral media containing 1 per cent thiosulfate were prepared essentially in the same manner described in a previous report (Starkey, 1934b). The initial reaction of the medium for *Th. novellus* and *Th. thioparus* was pH 7.8, for cultures B, T and K, pH 6.6 and for *Th. thiooxidans*, pH 6.2.

The data in table 1 indicate that polythionates were not produced by *Th. novellus, Th. thioparus*, or *Th. thiooxidans* at either early stages of growth or upon prolonged incubation. Cultures B, T and K show very evident reactions for polythionates. Similar tests have been repeatedly conducted with the numerous strains of the different cultures in media containing thiosulfate alone or together with organic materials, in media adjusted to different reactions and in the presence of various concentrations of salts and nitrogenous materials. Where growth developed,

TABLE 1

Production of polythionates by cultures in thiosulfate media

CULTURE	AGE	REACTION (pH)	IODINE TITRATION BEFORE KOH TREATMENT	IODINE TITRATION AFTER KOH TREATMENT
	days		*cc.*	*cc.*
Control	7	6.6	20.2	20.4
	26	6.6	23.7	24.3
B_1	7	7 2	15.9	21.5
	26	7.8	9.0	27.1
B_2	7	7.4	15.7	22.5
	26	7.6	12.4	25.8
T_1	7	8.2	8.1	25.7
	26	7.8	4.3	28.9
T_2	7	8.4	5.6	27.4
	26	7.6	2.7	29.1
K_1	7	8.4	6.0	28.0
	26	7.6	2.5	28.5
K_2	7	8.2	7.1	26.8
	26	8.0	6.4	25.7
Th. thiooxidans	7	6.2	18.8	18.9
	26	<1.2	0	0
Control	7	7.8	19.1	19.4
	26	7.8	22.4	22.7
Th. novellus (1)	7	7.6	18.6	18.7
	26	6.8	17.5	17.7
Th. novellus (2)	7	7.4	18.2	18.3
	26	6.6	16.9	17.1
Th. thioparus (1)	7	7.4	8.0	8.1
	26	5.0	0	0
Th. thioparus (2)	7	7.4	7.5	7.5
	26	5.6	0	0

the results with pure cultures were invariably in keeping with the data in table 1. In all cases where polythionates are produced by pure cultures the reaction becomes more alkaline as a result of oxidation of thiosulfate.

If all of the thiosulfate which was decomposed had been oxidized to pentathionate during growth, the titration with iodine after the treatment with KOH would not exceed that of the control solutions. If, however, either tetrathionate or trithionate was the product of oxidation, the iodine titration after KOH would exceed that of the control, more so from trithionate than from tetrathionate. This can be calculated from the data on a preceding page showing the distribution of sulfite and thiosulfate fron polythionates by alkali treatment, and from the fact that a unit of sulfite sulfur requires 4 times as much iodine for its oxidation to SO_4^{--} as one unit of thiosulfate sulfur to $S_4O_6^{--}$. It is therefore reasonable to expect that either tetrathionate or trithionate should appear in the end products of cultures B, T and K. The actual determinations for these constituents will be presented in a subsequent report.

Media containing elemental sulfur and supporting growth of *Th. thiooxidans* were also tested for polythionates by the method outlined above. In no instance was there any indication of the presence of such compounds. This is in agreement with previous studies with this culture (Waksman and Starkey, 1923) which indicated that there is no accumulation of intermediate products of oxidation of elemental sulfur.

TRANSFORMATION OF THIOSULFATE BY VARIOUS HETEROTROPHIC MICROÖRGANISMS

It appeared desirable to determine what influences known heterotrophic microörganisms might have upon thiosulfate, principally by reason of the fact that organic materials exert a pronounced favorable influence upon decomposition by cultures B, T and K (Starkey 1934b) and partly because there are almost negligible evidences of *growth* during attack on thiosulfate by these bacteria in mineral media. Three media were prepared. One was a mineral thiosulfate medium of pH 7.4. The second medium was like the first except that it received 0.2 per cent peptone and 0.2 per cent glucose. Medium three contained the organic materials but no thiosulfate. Eight representative cultures each of bacteria, actinomyces and fungi were used as indicated in table 2. The reactions of a few representative cultures are reported for media 1 and 3. The complete list is included for medium 2 since greater variations appeared in the medium containing both thiosulfate and the organic materials.

All cultures made good growth on medium 3, which contained no thiosulfate. There was no significant iodine titration or qualitative test for polythionates. The results, repeatedly verified, prove quite conclusively that neither the organic materials originally introduced in the medium nor the products of the microörganisms react with iodine under the conditions of the experiments sufficiently to affect the method used for detecting polythionates. In order to determine further the effectiveness of the method, a variety of solutions were tested. They contained either organic materials, thiosulfate, or prepared polythionates or combinations of these substances. The results clearly indicated that the method was very effective for detecting polythionates in the presence of the materials used in the culture media.

On medium one which contained thiosulfate but no organic materials, there was extremely limited growth of the actinomyces and fungi and no visible growth of the bacteria. On this medium the actinomyces and fungi made no significant change in pH, or decomposition of thiosulfate and the solutions gave no qualitative test for polythionates. Only 2 of the 8 bacteria decomposed thiosulfate. These two, *Pseudomonas aeruginosa* and *Achromobacter stutzeri* reacted in a manner typical of cultures B, T and K, producing polythionates and increasing the pH of the solutions.

In medium 2 which contained both thiosulfate and the organic materials, growth of all organisms was as extensive as on medium 3 showing no indication of any inhibitive effect of the thiosulfate. Only 2 of the cultures listed in table 2 decomposed large amounts of the thiosulfate. These are the same cultures that reacted upon the thiosulfate in the mineral medium. The organic matter

TABLE 2

Action of heterotrophic organisms upon thiosulfate

CULTURE	REACTION (pH)		TITER OF 5 cc. WITH 0.01 N IODINE		TITER OF 5 CC. WITH 0.01 N IODINE AFTER KOH TREATMENT	
	8 days	38 days	8 days	38 days	8 days	38 days
Medium 1. Thiosulfate alone						
			cc.	*cc*	*cc.*	*cc.*
Control	7.4	7.4	19.9	22.2	20.1	22.8
B. radiobacter	7.4	7.4	20 0	22.1	20.0	22.5
*Ps. aeruginosa**	7.4	7.6	19.2	18.8	20.3	23.6
*Achrom. stutzeri**	8.8	7.8	14.9	14.7	24.1	25.5
Act. bobili	7.4	7.4	19.5	21 8	19.7	22.4
Asp. flavus	7.4	7.4	19.7	22.1	20.0	22.4
Medium 2. Thiosulfate plus dextrose and peptone						
Control	7.4	7.4	20.0	21.5	20.6	22.4
B. radiobacter	7.4	7.6	20.8	21.6	21.0	22.1
Az. chroococcum	7.4	7.0	20.0	21.7	20.5	21.7
Cel. fima	6.2	7.6	20.3	21.4	20.6	21.6
Proteus vulgaris	6.2	7.4	19.8	20 1	20 2	20.8
*Ps. aeruginosa**	8.0	9.6	11.7	3.8	26.2	33.0
*Achrom. stutzeri**	9.8	8.6	6.8	0.9	30.0	28.2
B. cereus	5.4	5.4	19.6	20.1	20.4	21.5
Sarcina lutea	7.0	7.4	19.9	20.3	20.5	21.7
Act. bobili	7.2	6.8	19.3	21.7	20.6	22.1
Act. californicus	7.4	8.8	20.0	21.3	20.8	22.4
Act. flavovirens	6.8	7.8	20.1	21.3	20.7	22.3
Act. griseus	7.4	8.4	20.1	21.4	20.6	22.1
Act. Lipmanii	7.4	8.4	20 3	21.2	20.9	23.3†
Act. pheochromogenus	7.2	8.4	20.2	21.3	20.8	22.7
Act. purpeochromogenus	7.4	5.6	20.0	21.4	20.8	22.1
Act. violaceus ruber	6.6	8.4	19.9	21.1	20 6	22.0
Asp. flavus	7.4	7.8	20.3	21.9	20.4	22.3
Asp. niger	6.0	6.4	19.7	19.7	20.1	22.1†
Asp. oryzae	7.4	7.8	20.1	21.7	20.3	22.0
Penicillium sp	7.4	8.0	19.8	18.8	20.5	22.8†
Trichoderma sp	6.8	8.4	20.2	19.7	20.7	22.6†
Humicola sp	7.4	8.4	20.7	21.9	21.0	22.5
Mucor sp	6.4	7.4	20.6	20.1	20.6	23.1†
Rhizopus sp	6.2	6.8	20.1	19.4	20.2	22.9†
Medium 3. Dextrose and peptone alone						
Control	7.4	7.4	0.4	0.3	0.8	1.1
B. radiobacter	7.4	7.6	0.4	0.3	0.6	0.4
Ps. aeruginosa	7.8	8.4	0.4	0.4	0.5	0.5
Achrom. stutzeri	8.0	8.2	0.4	0.3	0.3	0.4
Act. bobili	7.4	8.0	0.4	0.4	0.8	0.5
Asp. flavus	7.4	7.6	0.4	0.4	0.3	0.2

* Active decomposers.

† Slight decomposition.

greatly favored their oxidation of the substance as it did the activity of cultures B, T and K. A few cultures other than the two bacterial species appeared to decompose slight amounts of thiosulfate. Wherever this occurred there was an indication that polythionates were produced, but these changes were slight.

In order to verify further the transformation of thiosulfate by heterotrophic bacteria, growth of the following cultures, obtained from the American Type Culture Collection, was studied on the three media described above: *B. subtilis*, *B. mycoides*, *Esch. coli*, *Ps. aeruginosa*, *Ps. fluorescens*, *Achrom. Hartlebii*, *Serratia marcescens*. All of the cultures made equally extensive growth upon media 2 and 3 but no visible growth on medium 1. *B. subtilis*, *B. mycoides*, *Achrom. Hartlebii* and *Serratia marcescens* failed to attack the thiosulfate and only one of these (*Achrom. Hartlebii*) is listed in table 3 as representative of the inactive forms. *Esch. coli* made slight development upon thiosulfate in the presence of the organic substances and apparently produced a small amount of polythionates. *Ps. aeruginosa* and *Ps. fluorescens* reacted upon thiosulfate characteristically both in the presence and absence of organic materials, being more active in the former case. As a result of their decomposition of thiosulfate, polythionates were produced and the pH of the media increased.

It thus seems that the transformation of thiosulfate is much more common among bacterial forms than has hitherto been acknowledged. It is interesting to note in this connection that, after careful cultural and morphological examination of his cultures, Trautwein concluded that they were similar to the fluorescent bacteria and were closely related to *Bacterium denitrificans* (Stutzer and Burri) L and N (1921; 1924). However, he believed that his cultures were autotrophic.

Although the various cultures which have been shown to have the capacity of decomposing thiosulfate and producing polythionates undoubtedly differ quantitatively in their activity, there seems to be no valid reason for assuming that the thiosulfate plays a different rôle in the nutrition of any one of these species than in others. It is reasonable to suppose that, as regards the transformation of thiosulfate, *Ps. fluorescens*, *Ps. aeruginosa*, *Achrom. stutzeri* and cultures B, T and K act qualitatively alike. This raises considerable doubt as to the autotrophic nature of any one of the organisms. Trautwein's claims for autotrophic development of his cultures depend principally upon the following two facts. The number of cells in the mineral medium lacking thiosulfate was found to be smaller than in the same medium with thiosulfate. In the second place, media kept free of carbon dioxide showed very slight decomposition of thiosulfate while media receiving carbon dioxide from the air showed active de-

TABLE 3

Decomposition of thiosulfate by heterotrophic bacteria

CULTURE	REACTION (pH)		TITER OF 5 CC. WITH 0.01 N IODINE		TITER OF 5 CC. WITH 0.01 N IODINE AFTER KOH TREATMENT	
	10 days	21 days	10 days	21 days	10 days	21 days
Medium 1. Thiosulfate alone						
			cc.	*cc.*	*cc.*	*cc.*
Control	7.2	7.2	19.5	20.6	19.8	20.8
Achrom. Hartlebii	7.2	7.2	19.4	20.1	19.6	20.2
Esch. coli	7.2	7.2	19.2	19.8	19.3	20.0
*Ps. aeruginosa**	7.8	7.8	15.9	15.6	22.0	22.0
*Ps. fluorescens**	7.8	7.8	16.3	16.1	21.0	22.2
Medium 2. Thiosulfate plus dextrose and peptone						
Control	7.2	7.2	17.5	18.5	18.1	18.8
Achrom. Hartlebii	7.2	7.2	17.5	17.8	17.6	18.2
Esch. coli	7.2	7.4	17.7	16.6	17.8	19.8†
*Ps. aeruginosa**	7.0	8.6	17.1	11.2	18.5	23.5
*Ps. fluorescens**	8.6	9.0	8.0	6.1	22.6	26.2
Medium 3. Dextrose and peptone alone						
Control	7.2	7.2	0.4	0.4	0.7	0.8
Achrom. Hartlebii	7.4	7.6	0.4	0.3	0.2	0.2
Esch. coli	7.4	7.2	0.4	0.4	0.3	0.4
Ps. aeruginosa	7.2	7.4	0.4	0.4	0.5	0.5
Ps. fluorescens	7.4	7.6	0.4	0.4	0.4	0.5

* Active decomposers.

† Slight decomposition.

composition. When organic substances were present, the transformation was active even in the absence of atmospheric carbon dioxide. Other explanations may be given for these observations; Trautwein's claims do not necessarily prove the autotrophic character of the bacteria. He even suggested that organic impurities in the solutions might have been responsible for the activity of the bacteria (1921, p. 525).

As a result of the present studies with the cultures of Trautwein's bacteria and other cultures which behave characteristically the same, it is most reasonable to conclude that these forms are not autotrophic. This judgment is based on the following observations: (1) Growth is much more active in media prepared from tap water than from carefully distilled water, particularly so if heavy inocula from nutrient agar slant cultures are used. As a result of this observation, tap water was generally used in culturing these bacteria. (2) Even in the media prepared as in 1 there is little or no actual visible *growth* of the cultures. Very apparent growth always characterizes development of *Th. thiooxidans, Th. thioparus* and *Th. novellus* under similar conditions. Particular care has been taken in the preceding discussions of organisms B, T and K to avoid the use of the term *growth* in connection with their decomposition of thiosulfate. There is little or no reason to believe that growth of the cultures is favored by the transformation of thiosulfate although thiosulfate is oxidized by reason of the presence of cells of the bacteria. (3) Organic materials which favor growth greatly increase the rate of oxidation of thiosulfate (Starkey, 1934b). This was not observed with the facultative autotroph, *Th. novellus,* or the strict autotroph, *Th. thioparus.* (4) Some other bacteria generally considered to be heterotrophic, attack thiosulfate in the same manner as Trautwein's cultures. Beijerinck also observed that *B. stutzeri* decomposed thiosulfate in an inorganic solution medium (1920), but his conclusion that the organism therefore had autotrophic characteristics finds no valid justification. (5) As will be shown in a subsequent report, no significant amount of carbon is assimilated by the cells from carbon dioxide and the specific reaction for the oxidation of thiosulfate yields a smaller amount of energy than is common to most processes displayed by autotrophic bacteria.

Until more convincing information is available to confirm the idea that these cultures can exist as autotrophs, it would seem most logical to conclude that the oxidation of thiosulfate to polythionates is brought about by these bacteria, while still developing as heterotrophs. It would not require a very strong oxidizing system to transform thiosulfate to tetrathionate. This appears to be the reaction which the bacteria bring about as will be shown in a later report.

Nathansohn found incompletely oxidized soluble sulfur materials in his thiosulfate medium supporting growth of *Th. thioparus* (1902). These materials were called polythionates and his complete theory of the mechanism of the transformation by the bacterium was based upon the assumption that polythionates were the initial products of oxidation. It is possible that some organism like those of Trautwein or a similar bacterium existed as a contaminant in the medium. At least, associations of such cultures as the *Th. thioparus* used in these studies and bacteria such as B, T, K or related organisms, form products from thiosulfate similar to those observed by Nathansohn, while a pure culture of *Th. thioparus* forms no other products than elemental sulfur and sulfate (table 4).

Some of the results of the studies with *Th. dentrificans* may find explanations for discrepancies from a similar interpretation. This is particularly applicable to Beijerinck's conclusion that his culture became transformed from an autotroph to a heterotroph by development upon organic media (1920). He almost invariably recovered denitrifying bacteria including *B. stutzeri* from his cultures. There is need for more complete details concerning the processes of oxidation of inorganic sulfur materials by carefully purified cultures of *Th. denitrificans* in view of conflicting results. Lieske found that some incompletely oxidized sulfur compounds could be detected in his cultural solutions (1912). His organism appeared to be a strict autotroph while a similar culture of Tjulpanova-Mossevitch appeared to be a facultative autotroph (1930).

Transformation of inorganic sulfur materials is not confined exclusively to the autotrophic bacteria and the heterotrophic forms considered in the preceding pages. Armstrong records that certain fungi decomposed thiosulfate in media containing carbohydrates (1921). Sulfate was the chief end product but hydrogen sulfide, sulfur and tetrathionate were also found in some cases. Guittonneau (1925 a and b; 1926; 1927 a and b; 1928) and Guittonneau and Keilling (1927; 1930; 1932 a and b) show that elemental sulfur can be transformed to thiosulfate by numerous heterotrophs including spore-forming and non-sporulating bacteria, by actinomyces and fungi. Some fungi also form sulfate from elemental sulfur. The reactions occur in solution media containing organic materials and in soils to which elemental sulfur has been added. In soils, they also found polythionates among the intermediate products of decomposition and generally obtained greater quantities of polythionates than of thiosulfate. These transformations were found to be due to biological action. Roach and Hobson (1930) also found polythionates and thiosulfate as intermediate products of oxidation of elemental sulfur in soil, with more of the polythionates than of the thiosulfate. The oxidation of thiosulfate in ammonia recovery liquor was found by Lockett to be due to biological action and, in the course of the transformation, large amounts of polythionates were detected (1913).

TABLE 4

Products of oxidation of thiosulfate by pure and mixed cultures of Th. thioparus

CULTURE	PER CENT OF DECOMPOSED THIOSULFATE-SULFUR RECOVERED					
	13 days			49 days		
	Sulfate-S	Poly-thionate-S	Elemental-S	Sulfate-S	Poly-thionate-S	Elemental S
Th. thioparus	60	0	40	60	0	40
Th. thioparus + B	53	12	35	50	22	28

In view of the fact that Kilpatrick and Kilpatrick found that thiosulfate used as a laboratory reagent decomposed as a result of bacterial action (1923), stock solutions of this type were tested in this laboratory, by the method discussed above, and were found to contain polythionates.

It is quite likely that other heterotrophic microörganisms than the cultures used in these studies are able to form polythionates from thiosulfate. However, some of these organisms such as *Achrom. stutzeri* and *Ps. fluorescens* are widely distributed in nature and may occur commonly in soil and water. Their activity in forming polythionates suggests that the polythionates, which occur where incompletely oxidized inorganic sulfur materials are undergoing decomposition, may be contributed in part at least from thiosulfate by the action of these or similar heterotrophic organisms.

The facts established during this series of reports recall to mind the recent interest in numerous organisms which cause some slight oxidation of ammonia or nitrite suggestive of nitrification (Nelson, 1929; Cutler and Mukerji, 1931; Cutler and Crump, 1933). Numerous heterotrophic organisms may be able to transform some amounts of mineral substances in ways suggesting development of autotrophs without necessarily altering our conception of the importance of autotrophic bacteria in the transformation of mineral substances in nature. The fact that numerous organisms may be concerned in reactions commonly ascribed to a few species, indicates the complexity of the changes occurring in associations of microörganisms in natural habitats.

SUMMARY

Two strictly autotrophic sulfur bacteria (*Th. thiooxidans* Waks. & Joffe and *Th. thioparus* Beij.) and one facultative species (*Th. novellus* Starkey) formed no polythionates during the oxidation of thiosulfate in solution media. Polythionates were not produced by *Th. thiooxidans* from elemental sulfur. Cultures of Trautwein (called *Th. Trautweinii* Bergey *et al.*), a similar bacterium isolated by the author (B), *Ps. fluorescens*, *Ps. aeruginosa* and *Achrom. Hartlebii* form polythionates from thiosulfate. Greater amounts of polythionates were produced in media containing organic materials which supported extensive growth of the bacteria. Numerous other bacteria, actinomyces and fungi which were tested failed to attack thiosulfate either in mineral media or in media containing organic substances. Thiosulfate

exerted no inhibitive effect upon growth of the heterotrophic organisms in concentration of 1 per cent.

It is concluded that: (1) The capacity to oxidize thiosulfate to polythionates is common to several heterotrophic organisms; (2) the bacteria of Trautwein (*Th. Trautweinii*), which have been called autotrophs are strict heterotrophs; (3) the appearance of polythionates in media or natural environments, where incompletely oxidized inorganic sulfur materials are undergoing oxidation, is probably the result of the activity of certain heterotrophic microörganisms which are able to form polythionates from thiosulfate.

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A PARTIAL PURIFICATION OF YELLOW FEVER VIRUS THROUGH ADSORPTION AND ELUTION

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There are now two processes available which may ultimately be adapted to the concentration and purification of the disease excitants classed as "filterable viruses." Ultrafiltration has been investigated extensively in this laboratory and a report on the method is now in preparation. The present paper describes a partial purification of the yellow fever virus by adsorption on kaolin with subsequent selective elution by dilute ammonia.

Adsorption on kaolin has been utilized successfully in the concentration of enzymes by Wilstaetter and Waldschmidt-Leitz (1923) and by Fordor, Bernfeld, and Schoenfeld (1925). Lewis and Andervont (1927) found that kaolin would remove the viruses of fowlpox and vaccinia from suspensions, but would not remove the excitant of chicken sarcoma. Callow (1927) was able to adsorb bacteriophage on kaolin and to release a portion from the kaolin by treatment with sodium carbonate, sodium sulphate, or di-potassium phosphate.

Kligler and Olitzki (1931) also adsorbed bacteriophage and fowlpox virus on kaolin, and subsequently eluted it with N/100 NH_4OH, obtaining over 50 per cent of the original bacteriophage content in a solution "free from even traces of protein." The Esbach test was used to detect protein. Rakieten and Hunt (1932) could not confirm their results. In a more recent publication Kligler and Olitzki (1934) report more extensive studies, and somewhat modify their original statements.

PRELIMINARY TESTS WITH YELLOW FEVER VIRUS

A preliminary test to determine whether yellow fever virus was adsorbed on kaolin and released by ammonia was performed as follows.

Experiment 1. Brains were removed from 5 mice exhibiting symptoms of encephalitis following the intracerebral inoculation of neurotropic yellow fever virus. These brains were ground in a mortar with 16 cc. of distilled water. The resulting emulsion was centrifuged at high speed for thirty minutes, the supernatant portion was then decanted off and filtered through a Seitz filter. To 9 cc. of the virus-containing filtrate was added 2.25 cc. of a 40 per cent suspension of kaolin in distilled water. The mixture was shaken for 1 hour at room temperature, then centrifuged. The supernatant was decanted off, passed through a small Seitz filter to remove any trace of kaolin, and was tested for the presence of virus by intracerebral inoculation into mice. The kaolin was resuspended in distilled water and distributed equally into four tubes and centrifuged. The supernatants were discarded. To the kaolin residues were added the substances indicated in the last four items of table 1. After standing one hour, tubes 4, 5, and 6 were again centrifuged and the supernatants were filtered and tested for virus. The suspension of kaolin in tube 3 was inoculated directly into mice. The results of the tests are given in table 1.

TABLE 1

Adsorption and elution of yellow fever virus

TUBE	CONTENTS	NUMBER OF MICE DYING	NUMBER OF MICE INOCULATED
1	Original filtrate	4	4
2	Original kaolin supernatant	0	4
3	Kaolin suspended in water	4	4
4	N/10 ammonia eluate	4	4
5	N/100 ammonia eluate	4	4
6	N/1000 ammonia eluate	0	4

The results with tube 2 indicate that all the virus present was adsorbed by the kaolin, while those with tube 3 show that the virus so adsorbed is infective for mice. The results with the remaining tubes indicate that the adsorbed virus can be released by ammonium hydroxide in concentrations of N/10 and N/100 but not N/1000. On this basis additional tests were made.

STUDIES ON ADSORPTION

The adsorptive capacity of charged particulate bodies varies with the concentration of motile ions in the suspending fluid. In biological systems these ions are for the most part H^+ ions; it therefore becomes of interest to determine whether a variation in hydrogen ion concentration within the limits permitting survival of the virus will affect the adsorption of virus on kaolin. This range of survival is approximately from pH 6.5 to 10.0, if a non-toxic buffer system, such as the glycine-acetate system (Northrup and De Kruif, 1922) is used. With phosphate buffers, this range is greatly reduced.

Experiment 2. Brains were removed from 10 mice showing symptoms of yellow fever encephalitis, ground thoroughly in a mortar, and suspended in 100 cc. of distilled water. The resulting emulsion was centrifuged for thirty minutes at high speed. The supernatant portion was decanted off and filtered through a Berkefeld "N" filter. This procedure was used in making routine virus suspensions, which are hereafter referred to as "stock filtrates."

To 5 cc. of this virus-containing filtrate were added 10 cc. of a glycine-acetate buffer of the required pH and 5 cc. of the stock 40 per cent suspension of kaolin in distilled water. Merck's NFV kaolin was used, and the suspension was sterilized by autoclaving. As a control on the toxicity for the virus, a similar series of tubes was set up with distilled water substituted for kaolin. The final pH of each mixture was determined electrometrically, since the addition of either protein or kaolin materially changes the pH value of the mixture, as is shown in chart 1. The mixtures were allowed to stand at room temperature for six hours, being shaken at frequent intervals. After this period the tubes containing kaolin were centrifuged, and the supernatant fluid was decanted off, passed through a small Berkefeld "N" filter, and tested for the presence of virus. The amount of protein was determined by a Kjeldahl test, correction being made for the amount of nitrogen in the buffer. The control tubes were similarly tested for the presence of virus. The results are shown in table 2 and in chart 2.

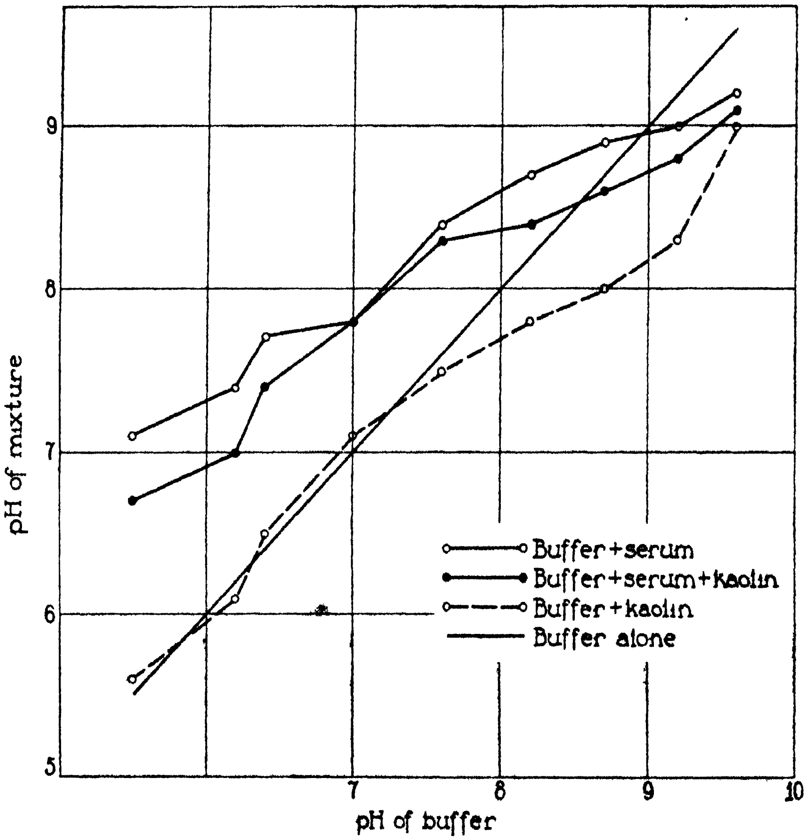

CHART 1. ALTERATIONS OF pH OF BUFFER MIXTURES BY SERUM AND KAOLIN (BUFFER 50 PER CENT, SERUM OR KAOLIN 25 PER CENT, WATER 25 PER CENT, BY VOLUME)

It is apparent that the virus is completely adsorbed throughout its whole range of survival. The amount of protein adsorbed, however, decreased with increasing pH values, as is shown in chart 2, the relation being linear in the short pH range covered. It appears that the control of pH at the time of adsorption is not important. In fact it was later found to be highly undesirable, since the presence of buffer partially inhibited the action of the eluting fluid.

TABLE 2

Survival and adsorption at varying pH

pH	VIRUS AND BUFFER		SUPERNATANT FROM VIRUS, BUFFER, AND KAOLIN MIXTURE		pH	VIRUS AND BUFFER		SUPERNATANT FROM VIRUS, BUFFER, AND KAOLIN MIXTURE	
	Number of mice dying	Number of mice inoculated	Number of mice dying	Number of mice inoculated		Number of mice dying	Number of mice inoculated	Number of mice dying	Number of mice inoculated
5.1	0	8	0	8	7.2	6	6	1	6
5.6	0	8	0	7	7.7	6	6	0	5
5.8	1	8	0	6	8.0	4	4	0	4
6.1	1	8	0	5	8.4	4	4	0	4
6.2	3	6	0	6	9.0	4	4	0	4
6.4	6	6	0	6	9.8	4	4	0	4
6.8	7	7	0	6					

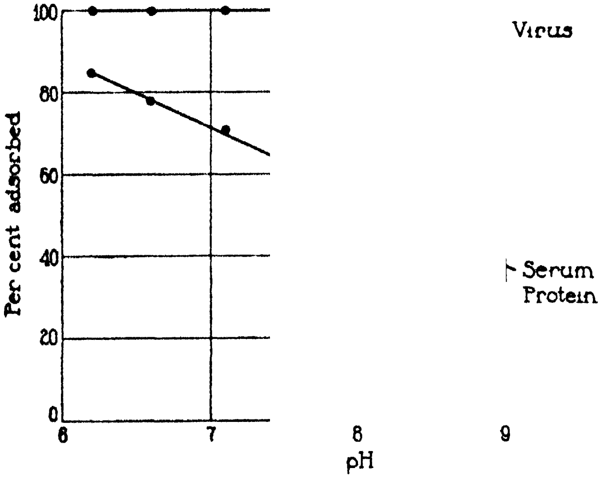

CHART 2. ADSORPTION OF VIRUS AND SERUM PROTEIN AT VARYING pH

Quantity of kaolin required

Experiment 3. Stock filtrate was prepared and mixed with 40 per cent kaolin suspension in the proportions shown (table 3). The mixture was allowed to stand for one hour with frequent shaking, and was then centrifuged. The supernatants were filtered through Berkefeld "N" filters and tested for the presence of virus. The results were as shown in table 3.

One-tenth volume of kaolin proved sufficient to adsorb all the virus from a suspension having a very low protein content, in this case about 5 mgm. protein per cubic centimeter.

TABLE 3

Quantity of kaolin required for adsorption

FILTRATE	KAOLIN SUSPENSION	NUMBER OF MICE DYING	NUMBER INOCULATED WITH SUSPENSION
cc.	*cc.*		
1	0.0	4	4
1	1.0	1	4
1	0.8	0	4
1	0.6	0	4
1	0.4	0	4
1	0.2	0	4
1	0 1	0	4

TABLE 4

Time required for adsorption

MATERIAL*	NUMBER OF MICE DYING	NUMBER OF MICE INOCULATED
Original filtrate	4	4
Supernatant, 2 minutes adsorption	3	4
Supernatant, 15 minutes adsorption	0	4
Supernatant, 30 minutes adsorption	0	4
Supernatant, 60 minutes adsorption	3	3

Time required for adsorption

Experiment 4. Stock filtrate was mixed with one-fifth its volume of the kaolin suspension, and samples were removed from the mixture at the time-intervals indicated in table 4. These samples were centrifuged as promptly as possible after removal, and the supernatants were tested for the presence of virus after filtration through a Berkefeld "N" filter. The results are shown in table 4.

The time required for adsorption is essentially that required to bring all of the suspended materials into intimate contact. In solutions of low concentration and low viscosity this contact should take place with rapidity, and such is found to be the case. In fact, when the contact is more prolonged than is necessary to remove all of the virus, there is apparently some dissociation, due possibly to replacement of virus by a more slowly adsorbed protein.

Effect of increased protein concentration

Unless the adsorption of virus is completely selective, the addition of other charged particles should result in a decreased capacity of the kaolin to adsorb yellow fever virus. To measure this effect the following test was made.

TABLE 5

Effect of protein concentration on adsorption

MATERIAL	NUMBER OF MICE DYING	NUMBER OF MICE INOCULATED
Original filtrate	4	4
Supernatant, 0 per cent protein solution	0	4
Supernatant, 10 per cent protein solution	0	4
Supernatant, 50 per cent protein solution	4	4
Supernatant, 95 per cent protein solution	4	4

Experiment 5. Stock virus filtrate was prepared in the usual manner. To portions of the filtrate, normal serum was added in such quantity that the final concentration of serum was 0, 10, 50, and 95 per cent of the original, while the amount of virus in each tube was uniform. These mixtures were treated with one-quarter of their volume of a 40 per cent kaolin suspension and allowed to stand for thirty minutes. They were then centrifuged, and the supernatants filtered and tested in the usual manner. The results are given in table 5.

This test indicates that one-quarter volume of kaolin (40 per cent suspension) will remove all the virus from a solution of protein containing the equivalent of a 10 per cent serum dilution (about 0.5 per cent) but is not effective in a protein concentration equivalent to a 50 per cent serum dilution (about 2.5 per cent protein). For satisfactory results, therefore, the protein content must be kept very low.

STUDIES ON ELUTION

Effect of various ammonia concentrations

The ideal concentration of the eluent is that which will release the maximum amount of virus without injury to the virus, and at the same time release the smallest amount of associated protein. An attempt to determine this concentration, utilizing the facts found in experiment 1 is described.

Experiment 6. Stock filtrate was prepared, and to 25 cc. was added 2 cc. of undiluted ascitic fluid to facilitate filtration and to increase slightly the protein content. One-quarter volume of 40 per cent kaolin was added, the mixture was centrifuged after standing for thirty minutes, and the supernatant was discarded. The kaolin was resuspended in distilled water, distributed equally into four tubes and again centrifuged, this supernatant also being discarded. The residual kaolin in each tube was then treated with sufficient ammonium hydroxide, of varying concentrations, to restore its original volume. After being mixed and standing for thirty minutes, the tubes were centrifuged and the supernatants were removed and filtered. Their virus content was then determined by intracerebral inoculation into mice, using 4 dilutions. The nitrogen content in the eluates was also determined by the Kjeldahl test after removal of all ammonia by aëration. The results are shown in table 6 and in chart 3.

TABLE 6

Effect of varying NH_4OH concentration on elution

MATERIAL	NUMBER OF MICE DYING/NUMBER INOCULATED				PER CENT DYING
	Undiluted	1:10	1:100	1:1,000	
Original filtrate	4/4	3/3	3/3	3/4	92.8
N/10 NH_4OH eluate	0/4	0/4	0/4	0/4	0.0
N/25 NH_4OH eluate	3/4	0/4	0/4	0/4	18.8
N/50 NH_4OH eluate	4/4	4/4	2/4	1/4	68.4
N/100 NH_4OH eluate	4/4	3/4	2/4	0/4	56.4

The maximum amount of virus was released at a concentration of N/50. Concentrations of NH_4OH greater than this apparently destroyed some virus. The quantity of protein released decreased with a decreasing concentration of ammonia. No protein was detectable by the Kjeldahl test when the virus was eluted with N/100 ammonium hydroxide; consequently, this concentration appears to be the optimal one for routine use.

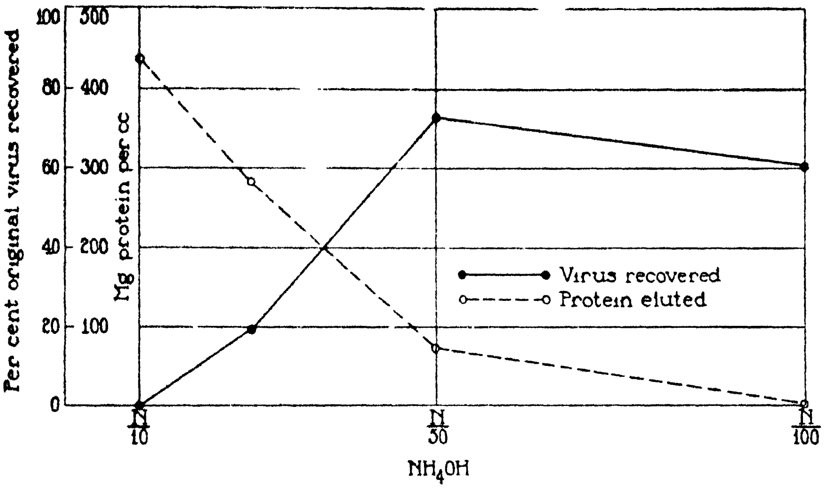

CHART 3. ELUTION OF VIRUS AND SERUM PROTEIN BY VARYING CONCENTRATION OF NH_4OH

Effect of time and temperature on elution

Kligler and Olitzki (1931) recommend elution at icebox temperature over night. Since this procedure results in the loss of the greater part of the yellow fever virus, tests were made to determine whether the process could be shortened.

Experiment 7. A stock filtrate was prepared and treated for thirty minutes with one-quarter of its volume of 40 per cent kaolin suspension. The mixture was centrifuged and the supernatant discarded. The kaolin was resuspended in distilled water and divided equally between three tubes, and again centrifuged. After discarding the second supernatant the tubes were brought to their original volume by the addition of N/100 NH_4OH. After being well mixed, they were allowed to stand for thirty minutes at the temperatures indicated. They were then centrifuged, and the supernatants filtered and their virus contents titrated. The results are shown in table 7.

There appears to be but little difference in results obtained at room temperature and icebox temperature. There is some slight evidence of an injurious effect at incubator temperature, since none of the mice inoculated with a 1:1000 dilution died.

TABLE 7

Effect of temperature on elution

TEMPERATURE	NUMBER OF MICE DYING/NUMBER INOCULATED				PER CENT DYING
	Undiluted	1:10	1:100	1:1,000	
°C.					
4	4/4	4/4	4/4	2/4	87.5
23	3/4	4/4	2/4	3/4	75.0
37	4/4	4/4	4/4	0/4	75.0

TABLE 8

Time required for elution

ELUTION TIME	NUMBER OF MICE DYING/NUMBER INOCULATED				PER CENT DYING
	Undiluted	1:10	1:100	1:1,000	
minutes					
2	4/4	4/4	4/4	4/4	100.0
15	4/4	4/4	4/4	4/4	100.0
30	4/4	3/4	3/4	1/3	73.2
60	4/4	4/4	4/4	3/4	93.6

Experiment 8. This experiment was designed to test the effect of various times of elution. Stock filtrate was prepared and its virus content titrated. It was then treated for thirty minutes with one-quarter of its volume of kaolin, after which time it was centrifuged and the supernatant fluid was discarded. Sufficient N/100 NH_4OH was added to restore the original volume and the suspension was well stirred. Samples were removed after two, fifteen, thirty, and sixty minutes exposure to ammonia. These samples were rapidly centrifuged, the supernatants were filtered, and their virus content was titrated. The results are given in table 8.

Two minutes appears to be sufficient for the completion of the elution process. The low figure obtained in the titration of the thirty-minute sample is obviously incorrect, probably due to the loss of virus in filtration, which is known to occur with some frequency.

From these results it appears that the yellow fever virus can be completely adsorbed out of solutions of low protein content—below 0.5 per cent protein—by adsorption with 40 per cent kaolin for a period of fifteen minutes. The virus so adsorbed can be released by treatment for two minutes with N/100 NH_4OH.

Composition of the eluate

Eluates prepared from mouse-brain extracts have consistently possessed a lower protein concentration than is demonstrable by by the Esbach, Adamkiewicz, or xanthoproteic tests. No protein could be demonstrated by the Kjeldahl test when using N/50 acid. The Molisch test for carbohydrates was also negative. On the contrary the biuret test has been strongly positive. On two occasions the volume of the eluate was reduced to one-fiftieth by distillation at 37°C. *in vacuo.* The material so concentrated also failed to give a positive protein test, and nitrogen could not be demonstrated by the Kjeldahl method. "Total solids" determinations have been made on three occasions, by drying 25 cc. of the eluate in a weighed dish over sulphuric acid, *in vacuo.* The values obtained were 0.00014, 0.00023, and 0.00041 gram per cubic centimeter. In all these preparations the virus content was such that 0.02 cc. of the eluate, when inoculated intracerebrally into mice in dilutions of 1:1000, would produce encephalitis. Since the original stock filtrate was made up in the proportion of 10 brains, of an average weight of 0.35 gram, in 100 cc., the final dilution in terms of original brain substance was 3.5×10^{-6}, disregarding loss by filtration or centrifugation.

When materials such as blood serum, with a higher protein content, are used for the source of virus, the final eluate will contain appreciably more protein. When infective monkey serum diluted with an equal volume of distilled water was used, the original protein content was 0.0319 gram per cubic centimeter.

When treated with one-half volume of kaolin, all of the virus, together with 11.9 per cent of the serum protein, was adsorbed. On elution with N/100 NH_4OH, 80 per cent of the original virus content was present in the eluate, which also contained 3 per cent of the original quantity of protein. By readsorption and a second elution, the amount of protein was reduced to a level below that demonstrable by chemical tests, and the final virus concentration was about 50 per cent of that of the original serum mixture.

DISCUSSION

It must be borne in mind that the limit of sensitivity of the chemical tests applicable to the demonstration of protein is reached in a dilution of about 1:10,000. On the contrary, the activity of the yellow fever virus is such that a 1 cc. dose of infective serum diluted 1:100,000,000 is frequently fatal to monkeys. Even this degree of activity is exceeded by the bacteriophage. Consequently, it is obviously unsound to state that such virus preparations are "protein-free."

It is apparent, however, that the virus particle behaves differently towards kaolin than do serum proteins, or other substances present in the blood serum or in brain extracts. It is more rapidly and completely adsorbed and, when so adsorbed, is released by a lower concentration of ammonia. That this reactivity may be due to a relatively high electric charge is suggested by the rapidity of migration of virus particles in an electric field.

Although the theoretical significance of these differences in reactivity is obscure, their existence offers a practical process for securing relatively pure virus suspensions for chemical and immunological investigations. In such preparations the protein content may be reduced much below the level of demonstrability with chemical tests, with no parallel loss in virus activity, as is well shown in table 8.

SUMMARY

Preparations of yellow fever virus have been made possessing a high degree of activity, and having a protein content at least fifty times less than can be demonstrated by chemical tests for protein. The method used is that of adsorption on kaolin followed by selective elution with dilute ammonia. This partial purification can be accomplished with ease and rapidity.

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A SYSTEMATIC STUDY OF MICROÖRGANISMS WHICH DECOMPOSE THE SPECIFIC CARBOHYDRATES OF THE PNEUMOCOCCUS[1]

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The soil bacteria which split the specific carbohydrates of the pneumococci differ in form and in cultural characters. The results of experimental studies on their immunological significance have already been reported (Sickles and Shaw, 1933, 1934). A systematic study of the different strains has been made in order to determine their relationships to each other and to known groups of soil organisms; this report records the results of that study.

The cultures described are of four distinct types:

1. Large spore-bearing rods which decompose type III pneumococcus specific carbohydrate, similar to those described by Dubos and Avery (1931); and, in addition, a strain which also utilizes agar.
2. Very small non-sporulating rods which attack the non-type-specific carbohydrate obtained from a degraded type I pneumococcus and the so-called "C" substance from typical strains.
3. Bacteria, oval in form, which decompose the specific carbohydrate of type II pneumococcus.
4. Slender rods with tapering ends, which utilize the specific carbohydrate of type I pneumococcus.

SOURCE OF STRAINS AND METHODS OF ISOLATION

With the exception of the very small non-sporulating rod which acts on the atypical I carbohydrate, of which we have only one culture, several strains of each of the above types were obtained from swamps and other uncultivated soils in different localities. One strain which decomposes the carbohydrate of pneumococcus type III was isolated from manure.

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[1] Presented before the Society of American Bacteriologists, Philadelphia, Pennsylvania, December 29, 1933.

Two kinds of mineral media were employed in the isolation and cultivation of these bacteria. Medium A was that described by Dubos and Avery (1931) in their report on the isolation of an organism which decomposed the capsular polysaccharide of type III pneumococcus. This consists of:

$(NH_4)_2SO_4$	1.0 gram
K_2HPO_4	2.0 grams
Tap water	1000.0 cc.

Reaction not adjusted—usually about pH 7.4.

Medium S has the following composition:

$MgSO_4 \cdot 7\,H_2O$	0.2 gram
$NH_4H_2PO_4$	1.5 grams
$CaCl_2$	0.1 gram
KCl	0.1 gram
$FeCl_3$	Trace
Distilled water	1000.0 cc.

10 cc. N/1 NaOH added per liter; pH from 7.2 to 7.4

To these mineral media were added the specific pneumococcus carbohydrates as a source of carbon, in concentrations varying from 0.002 to 0.01 per cent.

The soil samples were suspended in sterile water and inoculations made into flasks of mineral media containing the specific carbohydrates. These were incubated at different temperatures and under aerobic and anaerobic conditions. Since it was soon evident that no growth of the carbohydrate-decomposing organisms occurred under anaerobic conditions, later cultures were limited to those grown aerobically.

At intervals during the incubation, the cultures were tested for precipitation with the specific antipneumococcus serum. An absence of precipitation or a diminution in amount was the criterion for the presence in the mixed culture of an organism capable of decomposing the carbohydrate. As soon as precipitation was no longer obtained, the culture was transferred to fresh medium containing the specific carbohydrate. This served as a means of enrichment and to some extent eliminated extraneous forms unable to grow in these simple media. After a number of transfers in liquid medium, the carbohydrate of the medium was utilized in a shorter time, either because of the increased activity of the organisms or because of the continual elimination of contaminants. Blood-agar plates were streaked from these liquid cultures. Since the cultures which decomposed the type III pneumococcus polysaccharide were spore-bearers, heating to 80°C. before plating greatly facilitated their isolation. Colonies were fished into mineral medium containing the carbohydrate and the active fishings selected by the precipitation test. The cultures were then replated several times to assure their purity.

When the mixed cultures which decomposed the type II carbohydrate were plated on blood-agar or any agar containing beef-extract or peptone, all the fishings from these plates were inactive, indicating that the active organism failed to grow on these solid media. It was necessary to continue the process of enrichment and dilution until the variety of contaminants was so reduced that only a few types appeared when plated on beef-extract peptone agar. At this stage the enrichment cultures were plated on a semisolid agar made of mineral medium A, 0.8 per cent agar, and 0.01 per cent type II pneumococcus carbohydrate. Characteristic pink colonies appeared, which proved to be active organisms.

The mixed cultures, which utilized type I pneumococcus carbohydrate, contained, even after considerable enrichment, contaminants which overgrew the active organisms on all the solid media. The pure culture was finally isolated by continuing the dilution and enrichment procedures until the active liquid culture gave no growth when plated on beef-extract agar. This apparently pure culture was then plated on mineral medium S containing 0.8 per cent agar and 0.005 per cent type I pneumococcus carbohydrate. The small, pale yellow colonies developing slowly on this medium were fished and replated. The cultural and biochemical properties were then studied.

METHODS OF IDENTIFICATION

Standard media were prepared in accordance with formulae given in the Manual of Methods of the Society of American Bacteriologists (1932, 1933). Gram stains were made according to the Kopeloff and Beerman modification, and flagella were stained by Shunk's method, all as given in the Manual (1932).

In the study of the fermentation reactions of those strains which failed to grow on standard media, the test substances were added in 1 per cent concentration to mineral media. For the other strains, the action on carbohydrates was tested in the mineral media, also in Hiss serum water and in 1 per cent peptone solution.

For the determination of the optimum temperature, resistance to heat, and the limiting hydrogen-ion concentration, a medium previously found to be most favorable to the growth of the organism was used. This medium varied with the type of organism. Results were verified by using the mineral medium containing the specific carbohydrate. Growth of the organism under any given circumstance could then be determined not only by turbidity in the tube but also by decomposition of the specific polysaccharide. In the case of the strains which decomposed the type III or atypical I pneumococcus carbohydrate, inoculations were made from colonies grown on medium free from pneumococcus carbohydrate to avoid introducing any specific enzyme with the inoculum.

DESCRIPTIVE CHARACTERS

Bacteria which decompose the carbohydrate of pneumococcus type III

Morphology. Straight rods with rounded ends, occurring singly or in pairs. Vegetative cells 2.5 to 3.5μ in length by 0.6 to 0.8μ in width. Spores 1.5 by 2.5μ. Motile, possessing from four to six peritrichous flagella. Gram-negative. Figure 1 illustrates the vegetative cells and spores.

Colonies on blood agar or beef-extract peptone agar. Round, entire; usually about 2 mm. in diameter. Two types of colonies appear on plates—one white and opaque, the other gray and translucent. The white colonies produce spores; the gray ones are non-sporulating. This dissociation appears to be characteristic of all the strains studied, although it is more marked in some strains than in others. One strain utilizes agar, and colonies of this strain, while identical in appearance with those of the other strains, lie in depressions formed in the agar because of the softening effect on the gel. No liquid occurs in these depressions. Figures 2 and 3 illustrate the structure and type of colonies.

Fig 1 *Bacillus palustris* var *gelaticus* Bacteria from blood-agar plate after twenty-eight hours' incubation Gram Magnification × 1000

Agar slants. Moderate growth. Usually made up of colonies of both above types.

Potato slants. Growth heavy, viscid, yellowish, moist, and glistening.

Nutrient gelatin. No liquefaction.

Beef-extract peptone broth. Moderate even turbidity in twenty-four hours.

Beef-infusion broth. A less favorable medium than the beef-extract broth, at least forty-eight hours' incubation being necessary to promote growth. In old cultures, a heavy viscid sediment occurs.

Litmus milk. Litmus reduced. No digestion. No change in reaction.

Starch agar. Starch hydrolyzed weakly by two strains, strongly by the two other strains.

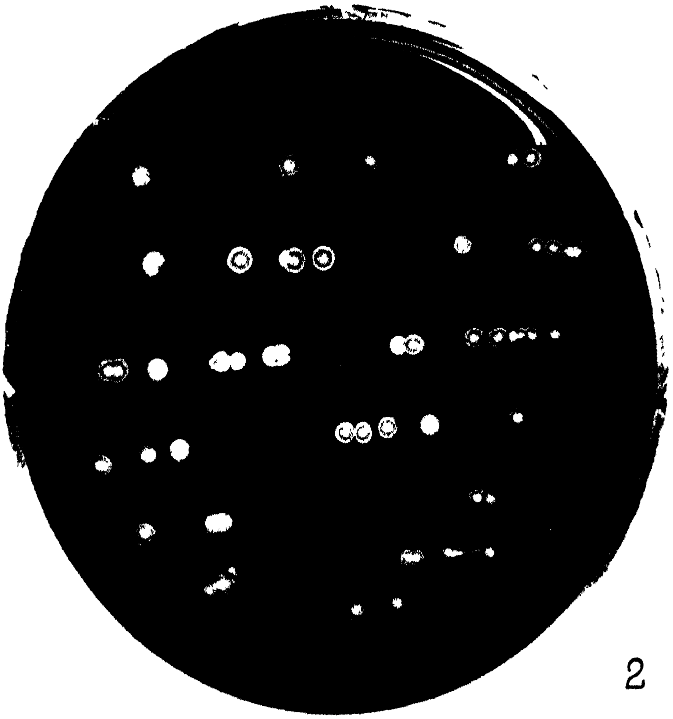

FIG. 2. *Bacillus palustris* var. *gelaticus*. Ninety-six-hour growth on blood-agar plate, showing colonial structure of sporulating type. Natural size.

Utilization of carbohydrates. Acid but no gas produced from glucose, lactose, sucrose, maltose, dextrin, mannitol, xylose, galactose, inulin, and salicin by all strains. Although glucose is utilized by the organisms, this carbohydrate has an inhibiting effect on the growth of the cultures if added to an otherwise favorable medium. Agar was utilized by only one strain: neither acids nor reducing sugars could be determined in the decomposition products, although the gel properties were obviously changed.

Relation to oxygen. Aerobic.

Temperature relations. Growth was obtained at from 22 to 42°C. Optimum temperature from 35 to 37°C.

Effect of reaction of medium. pH limits of growth from 5.5 to 8.5. Optimum not definite as the organism grew well over a considerable range. Usually grown at pH 7.2 to 7.4.

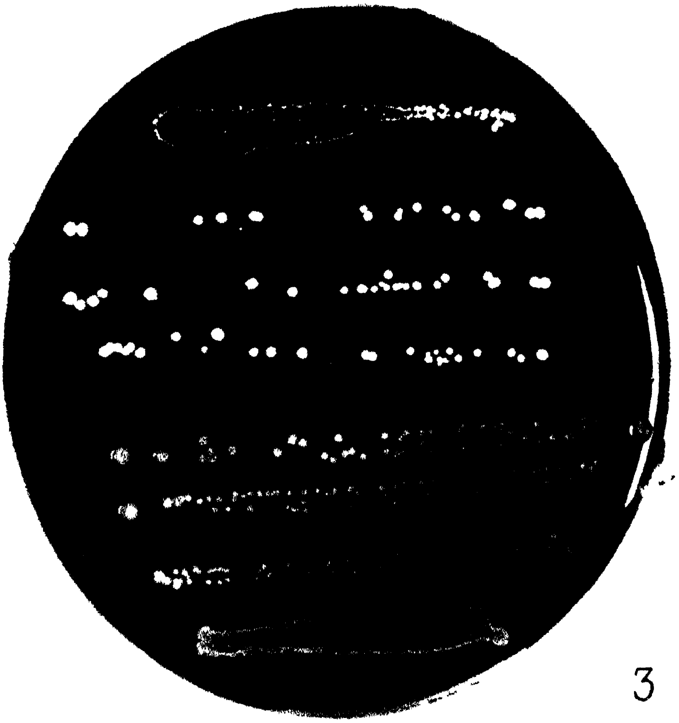

FIG. 3. *Bacillus palustris.* Differences in appearance of sporulating and non-sporulating colonies on blood agar. Natural size.

Bacteria which decompose the non-type-specific carbohydrate

Morphology. Small slender rods, occurring singly and in pairs, 0.7 to 1.0μ long. Width less than 0.5μ. No spores. Non-motile. Gram-negative.

Agar colonies. On blood agar, barely visible in twenty-four hours; after four days, dull rust-colored colonies about 1 mm. in diameter. Round, entire, umbilicate, and rather dry. On beef-extract agar, colonies are similar except that color is yellowish gray.

Agar slants. On beef-extract agar, very slight, thin, yellowish gray growth. On blood-agar slant, moderate growth; same color and texture as in colonies on blood agar.

Potato slants. Moderate growth, bright orange in color. Potato darkened.

Nutrient gelatin. At 37°C., gelatin cultures liquefied in one week. At room temperature, liquefaction slower, napiform; yellow sediment along line of puncture.

Beef-extract peptone broth. Moderate even turbidity. If non-type-specific carbohydrate is added to broth, a heavier growth occurs with yellow sediment.

Beef-infusion broth. No growth.

Litmus milk. After two weeks' incubation, a soft curd. Slight reduction of litmus. No change in reaction indicated.

Starch agar. Very active hydrolysis.

Utilization of carbohydrates. Acid but no gas produced from glucose, lactose, sucrose, maltose, dextrin, inulin; very slight action on mannitol; salicin not fermented.

Relation to oxygen. Facultative.

Temperature relations. Limits of growth from 22 to 39°C. No growth at 41°C. Optimum temperature from 35 to 37°C. Cells killed by heating for ten minutes at 52°C. Enzyme produced by strain against pneumococcus carbohydrate withstands 56°C. for ten minutes.

Effect of reaction of medium. pH limits of growth from 6.5 to 9.0. Optimum between 7.0 and 7.5.

Microorganisms which decompose the carbohydrate of pneumococcus type II

Morphology. Extremely pleomorphic. Young cells are oval in form, 1.5 by 2.0 μ, usually in pairs, and contain granules which stain deeply with basic dyes. Older cultures contain cells which may be from 12 to 15 μ in length. The granules in these occur in many different forms and arrangements. Nonmotile. Gram-negative. No spores noted. Figures 4, 5, and 6 illustrate some of the forms in which these organisms occur.

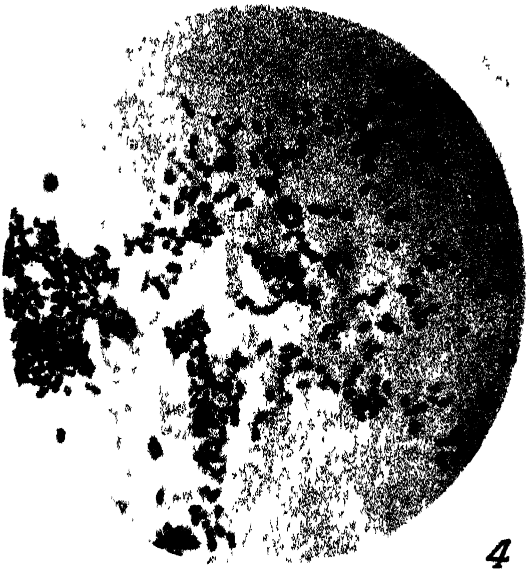

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FIG. 4. *Saccharobacterium ovale*. Young cells grown on sucrose semisolid agar. Methylene blue. Magnification, ×1000.

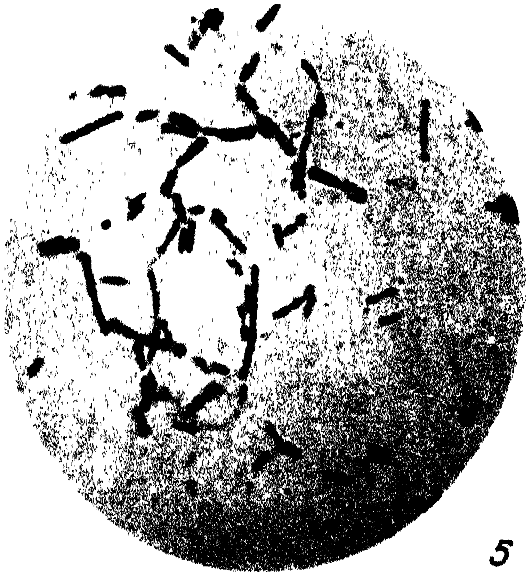

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FIG. 5. *Saccharobacterium ovale*. Seven-day culture grown on sucrose semisolid agar. Methylene blue. Magnification, ×1000.

Agar colonies. On beef-extract agar, no growth. On blood agar, no growth.

Medium A + pneumococcus II carbohydrate + 0.8 per cent agar. Very small round pink colonies pinpoint in size after about five days' incubation. After two weeks' incubation, they increase in size to 1 mm. in diameter. They are quite coherent so that, in fishing, whole colonies are removed from the agar plate.

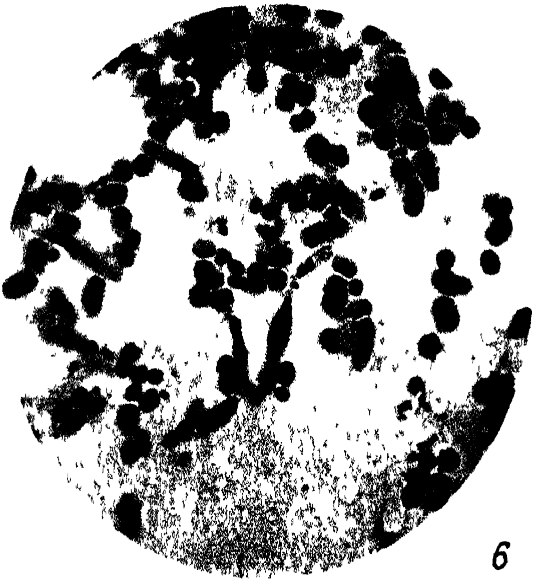

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FIG 6 *Saccharobacterium ovale* Seven-day culture grown in beef-extract peptone broth containing 1 per cent sucrose Methylene blue Magnification, ×1000

Beef-extract-agar slants. No growth.

Potato slants. No growth.

Nutrient gelatin. No growth.

Beef-extract peptone broth. No growth.

Beef-extract peptone + 1 per cent sucrose. Moderate turbidity. Yellowish sediment.

Beef-infusion broth. No growth.

Beef-infusion broth + 1 per cent sucrose. No growth.

Litmus milk. No growth.

Starch. No growth on nutrient agar containing starch. On agar plates containing mineral medium A and starch, there was a definite zone of hydrolysis.

Utilization of carbohydrates. In mineral medium A containing 1 per cent of the test carbohydrate, lactose and sucrose were utilized by all the strains, while maltose, xylose, and dextrin were decomposed by some of the strains. There was only a slight change in reaction. No growth occurred in the tubes containing inulin, mannitol, salicin, or 1 per cent glucose. When the concentration of glucose was reduced to 0.1 per cent, a slight amount of growth was apparent. Experiments on the addition of glucose to a favorable medium, such as the mineral base plus pneumococcus II carbohydrate, showed that glucose, even in 0.1 per cent concentration, had a marked inhibitory effect.

Relation to oxygen. Aerobic. In deep tubes of a semisolid medium, the growth of the culture is sharply delimited at 4 or 5 mm. from the surface.

Temperature relations. Limits of growth from 20 to 37°C. Optimum temperature from 34 to 35°C. Organisms are killed by heating for ten minutes at 54°C.

Effect of reaction of medium. pH limits of growth from 6.4 to 7.8. No growth at 6.0. Optimum pH from 7.0 to 7.4.

Effect of sodium chloride on growth. The addition of 0.5 per cent sodium chloride to any favorable medium completely prevents growth of the organism.

Microorganisms which decompose the carbohydrate of pneumococcus type I

Morphology. Extremely pleomorphic. Young organisms are pointed, often curved rods, 2 by 0.5 μ, having a densely staining granule in the central portion of the cell. The tapering pointed ends remain unstained. As the cultures become older, the ends of the cells become rounded, and in old cultures the organism may be a long oval or its form may be spherical or pear shaped. This change in form is probably associated with autolysis, as at this stage the cells stain weakly and subcultures usually fail to grow. The organisms are nonmotile and Gram-negative. No spores

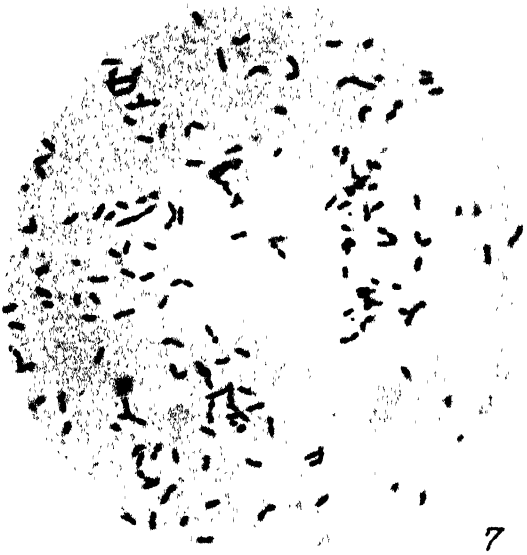

FIG. 7. *Saccharobacterium acuminatum*. Forty-eight-hour culture in medium S containing 1 per cent sucrose Crystal violet Magnification, ×1000

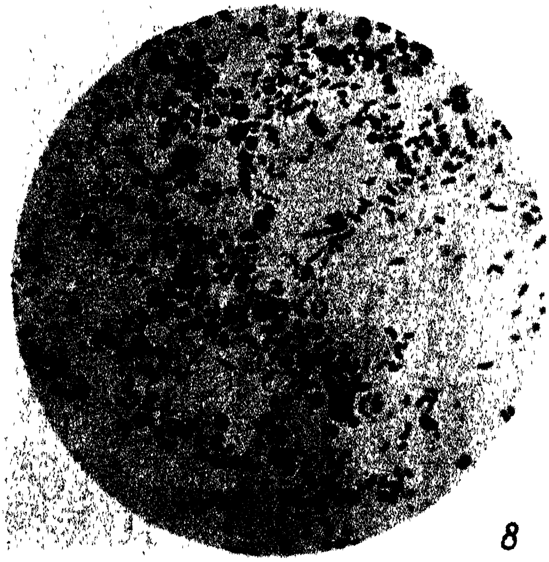

FIG. 8. *Saccharobacterium acuminatum:* Six-day culture on agar medium S containing 1 per cent sucrose. Crystal violet. Magnification, ×1000.

have been noted. Figures 7 and 8 illustrate some of the forms in which these organisms occur.

Agar colonies. On beef-extract agar, no growth. On blood agar, no growth.

Medium S + pneumococcus I carbohydrate + 0.8 per cent agar. Very tiny pale yellow colonies. Even after several weeks of incubation, they remain less than 0.5 mm. in diameter. On medium S containing 1 per cent sucrose and agar, the colonies develop more rapidly to 1 mm. in diameter, are pale yellow in color, and have the same texture as the colonies of the microörganisms which decompose the type II pneumococcus carbohydrate.

Beef-extract-agar slants. No growth.

Nutrient gelatin. No growth.

Potato slants. No growth.

Beef-extract peptone broth. No growth.

Beef-infusion broth. No growth.

Litmus milk. No growth.

Starch. Not hydrolyzed.

Utilization of carbohydrates. In mineral medium S containing 1 per cent of the test carbohydrate, the sucrose culture was the only one showing growth. Glucose, lactose, maltose, dextrin, inulin, mannitol, and salicin were not utilized. Further experiments indicated that glucose in 0.1 per cent concentration would support growth, but is somewhat inhibitory if added to an otherwise favorable medium.

Relation to oxygen. Aerobic.

Temperature relations. Limits of growth from 20 to 34°C. No growth at 37°C. Optimum temperature from 28 to 32°C. Organisms are killed by heating for ten minutes at 48°C.

Effect of reaction of medium. pH limits of growth from 6.0 to 7.8. Optimum pH from 6.6 to 7.2.

Effect of sodium chloride on growth. The addition of 0.7 per cent sodium chloride to any favorable medium completely prevents growth of the culture.

Effect of peptone and beef extract. The addition of 0.3 per cent beef extract or 0.5 per cent peptone completely inhibits growth.

Peptone in concentrations as low as 0.1 per cent has a deleterious effect on the development of the organism.

SPECIFICITY OF ACTION ON PNEUMOCOCCUS CARBOHYDRATES

With the exception of the strain which utilizes agar, the action of these organisms is quite specific when the pneumococcus carbohydrates are used as a sole source of carbon in a mineral medium. For example, the cultures which split the type II carbohydrate are unable to utilize the carbohydrates of pneumococci types I and III or the atypical I strain. It has been found, however, that, when additional sources of energy are furnished, the specificity is much less marked. Certain of the strains which split the type III carbohydrate may then utilize atypical I carbohydrate; most of those which split the type II carbohydrate will decompose the type III carbohydrate, but no strain except the pointed rods will attack the type I carbohydrates. It may thus be possible to broaden the activity of the other strains by cultivation in a suitable medium, and this point is now under investigation.

DISSOCIATION

An outstanding character of the strains which decompose the type III pneumococcus carbohydrate is their tendency to dissociate into sporulating and nonsporulating strains which produce characteristic colonies on beef-extract peptone agar or blood agar. This dissociation is spontaneous and is not permanent although subcultures will remain true to type for a short time before they in turn produce the two types of colonies.

This example of dissociation in a spore-forming rod seemed of especial interest because such distinctive characters as the decomposition of pneumococcus carbohydrate and the digestion of agar make it easy to determine that both types of colonies are variants of the same strain. Gee (1927) has described a similar type of dissociation in an organism resembling *B. vulgatus*, isolated from spoiled haddock.

CLASSIFICATION

The bacteria which decompose the carbohydrate of pneumococcus type III belong in the genus *Bacillus* but do not agree with the description of any of the species listed in that genus in Bergey's Manual of Determinative Bacteriology (1934). Although only a limited number of the more common forms of the genus *Bacillus* have been tested by us as to their ability to decompose pneumococcus carbohydrate, the evidence indicates that this particular character is unusual. We propose the name *Bacillus palustris* for these organisms.

A study of the descriptions of the species that digest agar, as given by Gran (1902), Biernacki (1911), Gray and Chalmers (1924), Aoi and Orikura (1928), Lundestad (1928), Angst (1929), Waksman and Bavendamm (1931), Goresline (1933), and Nichols (1933), indicates that the bacillus that acts on pneumococcus carbohydrate and agar differs from all the above in that it forms spores. Panek (1905) has described an organism which he named *Bacterium betaeviscosum.* Since the original of this paper was not seen, we do not know whether a definite statement was made concerning sporulation, but Panek's strain differs in another character. It is a facultative anaerobe.

The digestion of agar is relatively rare among the bacteria, but it is a question whether this character is of sufficient value to warrant placing in a different species the one strain which splits pneumococcus carbohydrate and also utilizes agar. It seems logical to consider this strain a variety rather than a species and to name it, therefore, *B. palustris* var. *gelaticus.*

Although we have not been able to identify it with any species described in Bergey's Manual (1934), the very small rod which specifically utilizes the atypical I carbohydrate may be classified in the genus *Flavobacterium.* We suggest the name *Flavobacterium ferrugineum* for this organism.

The relationship of the microörganisms that decompose the carbohydrate of pneumococcus types I and II to those organisms that digest cellulose and which are classed in the genera *Cytophaga, Cellfalcicula,* and *Cellvibrio* in the family Mycobacteriaceae is suggested by such characters as their form and pleomorphism, their failure to grow on standard media, and the inhibiting effects on growth of glucose, sodium chloride, and peptone. The toxic action of reducing sugars and of peptone on *Cytophaga* were noted by Hutchinson and Clayton (1919) and on a similar cellulose-digesting organism by Dubos (1928). Moreover, the picture presented by the cultures which act on the type I carbohydrate as they change from the pointed rods of young cultures to the slightly rounded form, similar in appearance to the young cells of the strains which act on the type II carbohydrate, and finally to the coccoid form of old cultures, resembles the series of changes recorded by Winogradsky (1929) and Krzemieniewska (1930, 1933) in the microörganisms which decompose cellulose. However, since no growth has been obtained with our cultures in liquid or on solid media with cellulose as a source of carbon, they obviously cannot be classed in the above genera.

Because of their apparent relationship to other genera in the family Mycobacteriaceae, we have classified them in this group. A new genus, *Saccharobacterium*, established on the basis of the utilization of the bacterial polysaccharides is proposed in which both strains would be placed because of their similarity in metabolic activities.

The name *Saccharobacterium ovale* is suggested for the strains which split the carbohydrate of pneumococcus type II and *Saccharobacterium acuminatum* for the pointed rods which decompose the specific polysaccharide of pneumococcus type I.

SUMMARY

Four types of microörganisms which decompose the specific and non-type-specific carbohydrates of the pneumococcus were isolated from soils and studied in detail. Methods for the isolation and purification of the cultures are given. The organisms are considered to be new species.

The name *Bacillus palustris* is suggested for those which decompose the specific carbohydrate of pneumococcus type III. The strain which also attacks the gel properties of agar is considered to be a variety, *gelaticus*, of the above species.

The name *Flavobacterium ferrugineum* is given to that microorganism which utilizes the non-type-specific carbohydrate obtained from a degraded type I pneumococcus.

A new genus, *Saccharobacterium*, in the family Mycobacteri-

aceae has been proposed for the microörganisms which decompose the specific carbohydrates of pneumococcus types I and II.

The name *S. ovale* is suggested for the strains which act on the carbohydrate of pneumococcus type II and *S. acuminatum* for those which decompose the polysaccharide of pneumococcus type I.

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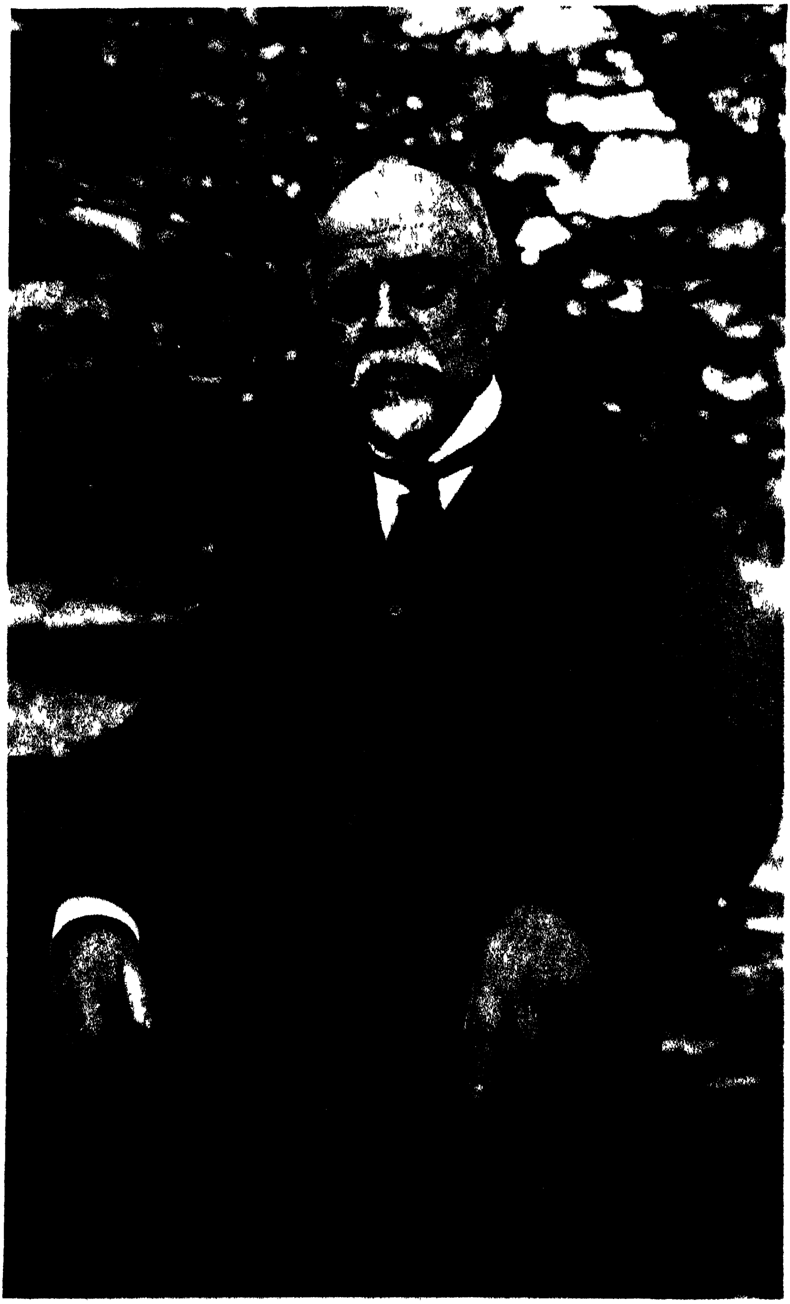

William Henry Welch

William Henry Welch[1,2]

1850-1934

Doctor Welch, the most honored and beloved leader of medical progress in this country, died at the Johns Hopkins Hospital in Baltimore on April 30, 1934.

William Henry Welch was born at Norfolk, Connecticut, on April 8, 1850. He came of a long line of distinguished rural physicians, the most notable of whom was his father, William Wickham Welch. His mother was Emeline Collin. Welch entered Yale College in 1866 and was graduated with the degree of B.A. in 1870. After his graduation from Yale he taught school for a year at Norwich, New York, but never deviated from his intention to follow the tradition of "The Doctors Welch of Norfolk."

In the fall of 1871 he matriculated as a medical student at the College of Physicians and Surgeons in New York. Perceiving at once his need for a firmer scientific foundation and perceiving also the inadequacies of the medical education of those days, he returned to Yale that year to study chemistry in the Sheffield Scientific School. Here he worked under able teachers, Professors O. H. Allen and G. F. Barker, and during this year he became associated with T. Mitchell Prudden, who was "filling a kind of voluntary instructorship."

Welch returned to his medical studies in New York in 1872. The didactic system of medical education of those days had many defects which he remedied in later years. But he always cherished "very fond memories of the College of Physicians and Surgeons." In a reminiscent talk on his sixtieth birthday he spoke particularly of his debt to two of his teachers there, Doctor

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[1] President, Society of American Bacteriologists, 1901.

[2] The photograph of Dr. Welch used as a frontispiece to this issue was very courteously placed at our disposal by Dr. Harvey Cushing of the Yale Medical School.

Edward Janeway and Doctor Francis Delafield. He said that he owed to Doctor Delafield his "first introduction to and interest in pathology." As an interne at Bellevue he spent much time in the "dead house" and in 1875, when he received the M.D. degree, he was far more deeply interested in pathology than in any other branch of medicine. Fortunately, in April of 1876 he was able to sail for Europe with his friend Doctor Frederic S. Dennis. During the next two years of study and travel in England and on the continent Doctor Welch worked with or met nearly all the notable medical men of that time. He was, therefore, in the midst of all the newest and most exciting developments in pathology and bacteriology.

After some training under Waldeyer in histology, von Recklinghausen in pathology and a brief meeting with Virchow, Welch had the good fortune to be admitted to Cohnheim's laboratory at Breslau. Here he investigated acute edema of the lungs, showing for the first time that pulmonary edema would occur when there was a disproportion between the actions of the right and left ventricles of the heart. The institute at Breslau was a stirring place. Ehrlich was there working with aniline dyes. Weigert also was studying the staining of cells. A. Neisser, Rosenbach and others who were to become famous were at work in the laboratory. Cohn was finishing his systematic investigations of bacteria. It was through Cohn that Robert Koch was invited by Cohnheim in 1877 to exhibit his new discoveries in anthrax. All the members of the staff watched those demonstrations of the growth, spore formation and pathogenic action of anthrax bacilli and shared in the enthusiastic appreciation of what Cohnheim declared were the "greatest bacteriological discoveries yet made."

When Doctor Welch returned to this country in 1878, rooms were given to him for a pathological laboratory at Bellevue. Now definitely launched on a career in pathology, he remained at Bellevue for the next six years, in spite of the precariousness of the livelihood. During these years he gained recognition as the outstanding representative of the new pathology in this country.

About this time, in 1884, the Johns Hopkins University, now in the eighth year of its fame, was beginning to form its medical faculty. Cohnheim's advice, confirmed by Doctor Billing's inspection of Doctor Welch at work, was followed by President Gilman in the selection of a Professor of Pathology. Doctor Welch was chosen. He accepted the offer in spite of inducements to remain in New York and the advice of friends that he would waste himself in remote and medically inconspicuous Baltimore.

Desiring first hand knowledge of the new developments in European bacteriology, Doctor Welch returned to Germany in the summer of 1884, to study under Koch. It was not until the spring of 1885, however, that he was able to take the course given by the great master. On Koch's advice he worked with others, first: with Frobenius, "a slavish teacher of Koch's technique," with Escherish, Buchner, Pettenkofer, Flügge, Nicolaier and many who became famous bacteriologists. Finally, he followed the course of one month in bacteriology taught by Koch in Berlin. Prudden who was there at this time has left a clear account of the calm, judicial mind of Koch and of his stimulation of his students "to personal exertion in this field and to lend certainty to the already widespread hope that ere long through the resources of science we shall be able to cope successfully with those most terrible and fatal enemies of the human race—the acute infectious diseases."

A mere list of the far-reaching enterprises fostered by Doctor Welch during the fifty years from the time of his appointment in pathology at the Johns Hopkins University in 1884 to the time of his death in 1934 would make a long catalogue. Much could be told about any one of his manifold activities. Any account of his life shorter than the full biography to be written by Doctor Simon Flexner must, therefore, be a selective description of some portion of his work. It will be necessary in this biographical note to consider particularly Doctor Welch's contributions to bacteriology and to pass over with briefer mention many things which were perhaps of more importance to him and to others.

Pathology was his first and abiding interest. He was the first in this country to "claim that pathology as a science, quite independently of any practical or useful applications whatever, is as legitimate and worthy an object of pursuit as any of the natural sciences." He had a broad and dynamic conception of pathology as the study of the natural history of disease. Of his pathological researches in Baltimore, his studies on thrombosis and embolism, glomerulonephritis, pneumonia and diphtheria were the most significant. In lectures, however, in demonstrations at the autopsy table, in brief reports on unusual cases, in comments and discussions he made innumerable additions to knowledge. His longer papers, published after years of research, are clear, thorough, scholarly expositions of their subjects.

"The central figure and guiding genius" among those who were establishing the Johns Hopkins Hospital and Medical School, Doctor Welch became increasingly concerned in medical education after 1886. With Osler, Halsted and Kelly in the hospital, which was ready for patients in 1889, and with Mall, Howell and Abel in the Medical School when it opened in 1893, he set the high standards expected of the students they hoped to attract and planned a new type of education. The essence of the new scheme was a reduction of professorial didacticism and the placing of the student in direct contact with problems, materials, patients and instructors. Doctor Welch, one of the most successful teachers, was aware of the danger that this new type of laboratory course might become restricted and limited in conception. He was never willing, therefore, to give up the lecture which he used to present the broad scientific, historical, scholarly and humanistic aspects of the subject. In an even wider field he exerted great influence in the direction of the extraordinary change which came over medical instruction in this country during his lifetime. The most significant of his final efforts was the part he played in the establishment of full-time professorships and full-time instruction in the clinical departments of the Johns Hopkins Medical School and elsewhere.

On numerous occasions, Doctor Welch spoke eloquently about the need for generous endowments of medical schools, teaching hospitals, laboratories and institutes for medical research. Perhaps no one has been as successful as he in enlisting the support of intelligent philanthropists for the best enterprises in modern medical progress in this country. Although he helped many, with generous impartiality, the two institutions closest to his heart were the Johns Hopkins Medical School and the Rockefeller Institute for Medical Research.

In the founding of the Rockefeller Institute in 1901, Doctor Welch's influence was of paramount importance. For the next thirty-three years he was chairman of its Board of Scientific Directors.

Always a teacher and spreader of new doctrines, Doctor Welch was from the time of his arrival in Baltimore until nearly the end of his life a keen and effective exponent of the newer preventive medicine. In 1889 he described the modes of infection to the physicians in Baltimore, telling them of the bearing of external sources of infection on preventive medicine. The outbreak of cholera in Europe in 1892 set him to lecturing on desirable sanitary reforms. The relation to public health of water supplies, sewage disposal and many phases of municipal sanitation were the subjects of several addresses. He exerted a most important influence in favor of the establishment of bacteriological laboratories in cities and states as a means of safeguarding the health of populations. During all this period he appears rather as an educator, an arouser of interest and stimulator of public opinion than a contributor to experimental knowledge of hygiene and sanitation, except for his very important applications of medical bacteriology. Doctor Welch served as president of the Maryland State Board of Health for twenty-four years, from 1898 to 1922. In the campaign against tuberculosis he delivered many addresses, and wrote many articles. A new adventure on which he embarked in 1916, at the age of sixty-six, was a natural outcome of these earlier years of interest in public health and hygiene. This was the creation and founding, under his directorship, of the comprehensive School of Hygiene and Public Health of the Johns Hopkins University. He remained at its head during the next ten years, until 1926.

At the age of seventy-six, as Doctor Cushing said, "he might have been allowed to approach in quiet the evening of his days, content with the many successful parts he has already played," but it was "not his way" to turn aside from a new venture. In 1926, he accepted the Professorship of the History of Medicine at Johns Hopkins, newly endowed by the General Education Board. Other gifts soon made possible the building of the beautiful William H. Welch Memorial Library, the consolidation of three great collections of books, the gathering of a store of historically valuable material, and the inauguration of the Institute of the History of Medicine under Doctor Welch's guidance. Always an omnivorous reader, keenly studious in history and literature, broadly cultured, Doctor Welch satisfied another lifelong interest in this new undertaking. The Library and Institute, under him and his brilliant successor, Doctor Sigerist, have already served high purposes of scholarship.

While he was President of the National Academy of Sciences, from 1913 to 1916, Doctor Welch did much to supply the government with information on scientific resources for waging war and was one of those who helped to place the National Research Council at the service of the departments in Washington. His own war service seems to have begun at a visit to the office of the Surgeon General, in 1917. It is said that when he saw the pile of correspondence on General Gorgas' desk he asked if he might not be of use in "opening the mail." Commissioned first as a major in 1917, he was soon promoted to the rank of colonel and in 1921, when he left the medical reserve corps, he was a brigadier general. He had a distinguished record as an advisor to the medical department of our army.

The story of Doctor Welch's contributions to bacteriology has been postponed until near the end of this account of his life in order to permit the consideration of all those activities which affected not only the establishment of departments, schools and institutes, but also the beginnings and growth of a new biological science. Furthermore, the part he took in this work can be understood more clearly after some comprehension of the man himself has been gained.

Bacteriology had its protagonists in the United States even before Doctor Welch saw Robert Koch's demonstration of the anthrax bacillus in Cohnheim's laboratory in Breslau in 1877. The art and science of bacteriology in this country has not yet found its adequate historian, but it is certain that the new knowledge of germs was coming into the medical centers on this side of the ocean while William Henry Welch was still at school in Norfolk. Medical observers in France sent back news of Pasteur's discoveries. There was, of course, an interchange of scientific communications in journals. A southern physician who took his microscope with him during the Civil War and examined the intestinal contents and Peyer's patches of soldiers dead of typhoid fever, saw bacteria in them. In New Orleans, about 1874, a physician, who was familiar with Davaine's work, attempted to produce yellow fever in guinea pigs by inoculating them with black vomit and putrid materials. He was convinced that the cause of yellow fever was a *contagium animatum*. Sternberg at about the same time also believed in the zymotic cause of yellow fever. The New Orleans Medical and Surgical Journal published many papers and reports on medical bacteriology during the years before 1880, including an account of the application of Lister's methods of antiseptic surgery in the wards of Charity Hospital in New Orleans in 1874–75. Sternberg was sufficiently familiar with bacteriology in 1880 to discover the pneumococcus in his saliva. In 1878 Burrill made his brilliant discovery of the microbial cause of the blight of apples and pears. No doubt medical journals of many communities contained much instructive bacteriological information, together with exposition of general ideas concerning infection, derived from French, German and English sources, before the pupils of Robert Koch brought the new technic and the postulates to America.

If, however, the beginnings of precise bacteriology may be dated from the time when an investigator could be certain that he had pure cultures of bacteria to study, then Doctor Welch, who learned these new methods from Koch himself within three or four years after their invention, was among the first practitioners of scientific microbiology. Welch, Prudden, Salmon, Theobald Smith, Sedgwick, Burrill and a few more who should be mentioned if the record were complete, composed this group of pioneers.

To skill in the newer procedures Doctor Welch added a surpassing comprehension of the significance of the medical applications of bacteriology. He was an active cultivator of this field during the exhilarating decade, 1880–1890, which was, he said, "perhaps the most wonderful decade in the history of medicine" because it produced "a revolution in medical thought through the discovery of the agents causing infectious diseases."

Doctor Welch's contributions to bacteriology may be grouped in three classes: first, the results of his productive investigations; second, the effects of his teaching in the training of bacteriologists, the education of physicians through addresses at their meetings and the instruction of the general public; third, the material and intellectual aid he gave through his part in the establishment of laboratories and institutes in which some of the most fundamental investigations of the bacteria, viruses and protozoa have been made.

His early papers in bacteriology were broadly educational. The first appears to have been an address in 1887 on modes of infection. During the years after this he presented a number of scholarly reviews of such subjects as preventive inoculation against infection, the relation of bacteria to pneumonia, the bearing of external sources of infection on preventive medicine, the new knowledge of diphtheria, a general discussion of immunology, and a whole series of addresses on tuberculosis. It would be difficult to over-estimate the clarifying effects of these lucid and instructive surveys at a time of confusing and conflicting publications in a rapidly developing picture of the biology of infectious processes. This is not the place in which to attempt to catalogue the successions of his pupils who became notable in bacteriology. His effect upon them was indelible. Nor is it possible to estimate the indirect contributions he made by apt suggestion. An example of this is his advice to Walter Reed that it would be important to test the filtrate of the blood of a yellow fever patient.

Although Doctor Welch started his investigations in bacteriology soon after his arrival in Baltimore in 1885 it is difficult to assign a date to the first publication of his original observations. It is more than probable that brief reports of his own work are hidden in general statements in longer dissertations on pathological subjects. An example of this is to be found in the Cartwright lectures on fever which he delivered in 1888. There, in two sentences, he tells of his isolation of the tetanus bacillus, though not in pure culture, from the soil of Baltimore and its neighborhood.

His first formal bacteriological paper is the report of his studies of hog cholera and swine plague, published in 1889. In this and a subsequent paper in 1894 he confirmed the findings of Salmon and Smith, differentiating the organisms associated with those two types of infection.

The etiology of pneumonia and the pneumococcus were thoroughly investigated by him during the years 1890 and 1892. The papers he published on these subjects are models of their kind. Variations in virulence of the pneumococcus interested him a great deal. His papers on the pneumococcus in 1892 contain descriptions of the Welch capsule stain, a modification of Guarnieri's medium, a wealth of observations on experimental infections and an excellent systematic account of the pneumococcus and its history. Even at this early date he investigated the possibilities of therapy or protection with antipneumococcus serum.

During the same years, 1890 to 1892, he investigated the etiology of diphtheria. At that time, Prudden's failure to find diphtheria bacilli in supposed cases of diphtheria in New York was retarding the acceptance of Löffler's discovery. With Abbott in 1891, Doctor Welch published a paper confirming Löffler's work and gently correcting Prudden. He emphasized the value of rapid bacteriological diagnosis of diphtheria. In 1892, he published with Doctor Simon Flexner two important papers on the histological changes caused by the "toxalbumen of diphtheria." Although two later papers on diphtheria do not contain accounts of more of his original observations, they were very influential in their time in crystallizing the new knowledge of this disease and in establishing antitoxin therapy. The first of these in 1894 was a survey of diphtheria in the United States; the second, in 1895, was a most able and completely documented general and statistical presentation of the subject of the treatment of diphtheria by antitoxin.

Conditions permitting the invasion of tissues and blood stream by colon bacilli were investigated by Doctor Welch in 1891. In general, he regarded this organism as of little pathogenic importance, attributing its presence usually to post-mortem or agonal invasion.

In 1891 also he reported experiments on the production of the typhoid carrier state in rabbits. Apparently, typhoid bacilli, and indeed many of the organisms causing acute infections of the intestines, were studied by him during this time. A significant outcome of this phase of his work was an authoritative paper on the serum diagnosis of typhoid fever, published in 1897.

He began, in 1891, to publish the results of many years of work on skin sterilization, and surgical infections, the outcome of a fruitful collaboration with Halsted and others. His article "General Bacteriology of Surgical Infections," published in Dennis' System of Surgery in 1895, is a mine of information. It contains further comments on the significance of the special variety of the white micrococcus which he discovered in the skin in 1891 and had named *Staphylococcus epidermidis-albus*. A section of this article on "Bacterial Association" shows his comprehension of processes now called antagonism and synergism and his realization of the fact that a theory of mono-etiology of some diseases might not be adequate, because "mixed infections are common in human beings."

The studies with which his name as a bacteriologist is most commonly identified began in 1891 when he performed an autopsy on the body of a man who had died from rupture of an aneurysm. Doctor Welch found gas in the blood vessels of the cadaver and discovered that the gas was produced by an anaerobic bacillus not known before that time. A brief report on this case in 1891 was followed in 1892 by the comprehensive publication of the research conducted in collaboration with Nuttall, in 1896 by the thorough study carried out in association with Doctor Simon Flexner, and in the same year by his Shattuck lecture on morbid conditions caused by *Bacillus aerogenes-capsulatus*. In 1900, at a meeting of the Society of American Bacteriologists, he read a brief report on the distribution of the gas bacillus. In this last report

Doctor Welch placed the name *Bacillus Welchii*, Migula, in parenthesis in the title for the first time. Most of the essential knowledge of the Welch bacillus was discovered in the course of these investigations. Two characteristics of importance, however, not noted then were the formation of spores, which were demonstrated by Fraenkel in 1893, and the production of a soluble toxin, which was investigated with beneficial results by Bull and Pritchett in 1917, when, during the World War, the Welch bacillus was recognized as one of the most serious and common causes of gas gangrene in wounded soldiers.

Of the discoveries which Doctor Welch made and handed on to others for investigation there will never be a complete record. One example of this is his remarks on blastomycosis in the course of a discussion before the Association for the Study and Prevention of Tuberculosis, in 1908. In recalling the first observations on blastomycotic infections in this country he said that in 1893 he received "from Doctor Rixford, of San Francisco, material excised from a patient with a singular affection of the skin. In this I discovered a peculiar parasite and I subsequently turned the material over to Dr. Gilchrist, who in conjunction with Dr. Rixford published the case in full." It is evident from the rest of this account that if he had cared to seize the opportunity he could have secured priority in the description of the American varieties of *Blastomyces* and *Coccidioides*.

Doctor Welch was a learned and forceful expounder of current bacteriological and immunological theories, but he seems rarely to have expressed an hypothesis of his own. From the bacteriological point of view, his most interesting announcement of an hypothesis was made in the course of his Huxley lecture on immunology at Charing Cross Hospital Medical School in London, in 1902. After pointing out the manner in which the cells of the animal body defend themselves against bacteria by producing antibodies he said: "I see no reason why suitable substances derived from the host may not stimulate parasitic organisms, through a physiological mechanism similar to that operative in the development of cytolytic immunity, to the production of intermediary bodies which if provided with the requisite affinities, have the power to link complements to cellular constituents of the host, and thereby poison the latter." This hypothesis of a bacterium defending itself by producing antibodies against its host, a fundamental problem in parasitism, was in Doctor Welch's opinion, susceptible to experimental test. Further investigations on it, however, were not published by him.

It is apparent from this review of Doctor Welch's researches that his chief interest lay in the application of bacteriology to the solution of problems of infectious disease. He was less conspicuously associated with studies of the bacteria on account of their own intrinsic and unique importance than he was with investigations of what they do to man and animals. He did not claim for bacteriology as he did for pathology the right to be recognized as an independent science. From these facts the inference might be drawn that Doctor Welch was not deeply concerned in the development of the general field. It would be a mistake, however, to stress that deduction, because his interests were always wide-ranging and because many fundamental contributions to the biology of microörganisms have come from his pupils and from investigations conducted under his influence. According to his definition of a science bacteriology was qualified for that recognition. He said at the dedication of the Hull Biological Laboratories in 1897: "Whenever a body of scientific knowledge has reached a state of development in which its extent is considerable and its problems and methods of attacking them are special, it is convenient and proper to recognize it as a branch of science whose interests will be best furthered by workers specially trained to its service." At the same time Doctor Welch expressed his opposition against too much specialization and at the Johns Hopkins Medical School he retained bacteriology as a subdivision of pathology. The essence of his attitude toward bacteriology, however, is expressed in his introduction to the first volume of the Journal of Experimental Medicine. Here he wrote, in 1896: "The epochal discoveries of the causal relations of microörganisms to infectious diseases have opened a fruitful and important field of research. No medical science is today cultivated more zealously and with more significant results than that of bacteriology, ..."

When the Society of American Bacteriologists was formed in 1898, Doctor Welch was withdrawing more and more from the laboratory in order to meet the demands made upon him by general medical affairs. He was occupied with medical education, the antituberculous campaign and a multitude of activities, among which in 1898 was his extraordinarily able defense of animal experimentation and his defeat of the antivivisection bill before the Senate of the United States. His connection with the Society of American Bacteriologists was never a very intimate one. He was elected the second President of the Society and served in 1901. Later he was made the first Honorary Member of the Society. One who recalls his presence at the Society's sessions has written: "None of us will ever forget the occasions on which he did attend our meetings and contributed to our discussions with his characteristic grace and inimitable illumination of every subject which he discussed."

Doctor Welch's versatility was so manifold and his accomplishments were so numerous during his long and kindly life that only those with the gift of eloquence can summarize his career or portray his personality in a few words. From one who must have known him well, an anonymous author of one of the tributes given him at the celebration of his eightieth birthday, a partial quotation may be used to express universally the manner of remembrance of Doctor Welch:

"To have stepped, in the prime of life, into a position of acknowledged intellectual leadership in the profession of his choice; to have occupied that position, albeit unconsciously, for those . . . . years which have seen the most rapid strides in medical progress of all time; to have had such influence in the furtherance of the medical sciences in this country as to turn the tide of students seeking opportunities for higher education from the Old World to the New; to have been as ready in countless unrecorded ways to share his time with those who were inconspicuous as with those who sat in high places; to have been no less universally respected for his great learning than beloved for his personal charm and companionability; to have stood knee-deep in honors unsought and to have remained seemingly unaware of them; . . . . To have done so much, in so many ways, for so many years, and to have aroused no shadow of envy or enmity on the way, betokens not only unselfishness of purpose but that fineness of character which always has been and always will be an inspiration to mankind."

S. BAYNE-JONES.

ON THE ENZYMES OF BACTERIA AND BACTERIAL METABOLISM[1]

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The important class of bacteria includes an immense number of forms and types of living cells, wonderfully adapted to varying environments: from obligate aerobes to obligate anaerobes; from types growing at the temperature of zero to thermophilic groups; from strict autotrophs to strict metatrophs; etc. This rich variety in growth requirements makes the bacterium an excellent object for metabolism and enzyme studies.

The formation of enzymes in living cells is undoubtedly one of the fundamental problems of biochemistry. Regarding, as we do, the metabolism of the cell as a resultant of numerous enzymic reactions, we may properly recognize the enzyme action of the cell as characterising the species, especially when dealing with bacteria whose morphologic characteristics cannot be clearly ascertained. The newer bacteriological literature contains frequent statements of a transformation of one species to another among the bacteria. Such a remarkable phenomenon would mean that the bacterial cell could easily change its enzyme action by producing entirely new enzymes or discontinuing the production of others, and that, consequently, the classical idea of the consistancy of bacterial species would have to be dropped. Unfortunately these investigations fail to give reliable proofs with reference to the real gist of the matter: has a transformation actually taken place, i.e. has the enzyme action of a given bacterial species been substantially changed?

Our investigations, in which the enzyme action of bacterial cells was quantitatively followed under different conditions, have led to the following results:

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With regard to the important metabolic enzyme *catalase*, quantitative experiments (Virtanen and Karström, 1925; Virtanen and Winter, 1928) with a laboratory strain of *Bacterium coli* showed that the catalase activity of a cell is wholly independent of the food supply. When *Bact. coli* was grown on ammonium salts and glucose its catalase activity was quantitatively the same as when the nutrient solution contained complex organic nitrogenous compounds, such as peptones, etc. From this it can be deduced that the catalase is built up by the cell from the simplest building materials. Neither was the amount of catalase in a cell dependent upon the temperature or the acidity of the medium. The catalase activity of the strain of *Bact. coli* has been remarkably constant, remaining unaltered during the ten years this strain has been used in our laboratory.

The constancy shown by catalase is however by no means common to all enzymes. In earlier literature attention is frequently paid to the fact that microörganisms are able to produce certain enzymes only if grown in the presence of the substrate specific for the enzyme in question. Observations of the above kind have led many workers to assume that the cells could be "adapted" to form almost any kind of enzyme, in which case the transformation of bacteria could also be easily understood.

This is, however, a false assumption. "Adaptation" certainly widens the scope of enzyme action but only in accordance with well-defined rules. Our work in this direction, as described especially by H. Karström (1930) in his dissertation, shows that a given strain of a bacterium can only be "adapted" to form given enzymes. The ability to form certain enzymes is thus a characteristic of a given strain. Irrelevant new enzymes have never been produced by the microörganisms under investigation. Karström suggests the term "constitutive" for the enzymes produced by a given strain irrespective of the kind of nutrition, and "adaptive" for those produced by a given strain only in the presence of the specific substrate of the enzyme. As mentioned above, even the adaptive enzymes of a given bacterial strain are strictly defined, and will thus never give rise to the formation of new forms with new characteristics.

The real cause of the formation of certain enzymes through "adaptation" is still problematic. Hypothetical explanations can naturally be put forward: for example, by assuming that even the adaptive enzymes are always present in the cell but will become active only when the cell feeds on the specific substrate in question. Such explanations are however not supported by experimental evidence. In general it seems that the metabolic enzymes proper are always constitutive, whereas, the enzymes (especially hydrolases) which convert the nutrients into suitable form for the actual metabolic reactions may be adaptive.

In the course of our investigations we could not find a single case in which bacteria have in fact produced new enzymes or in which the enzymes did not belong to the usual enzyme-system of the strain. The only case with a seeming change in the enzymic action of a strain was the loss of the gelatin-liquefying property by *Bacillus fluorescens-liquefaciens* over long periods, when the gelatin-cultures were allowed to dry before an inoculation into a fresh medium was made. This observation corroborates Matzuschita's earlier report (1914). As will be seen later on, the property to liquefy gelatin depends upon the ability of bacteria to excrete proteinase. Consequently, the loss of liquefying property does not mean that the cell has ceased to *form* proteinase, but that for some unknown reason it is unable to excrete it.

Up to the present we have not studied the question whether the enzyme-system of the bacterial cell may be permanently influenced by certain agents like ultraviolet or Röntgen-rays. On the basis of our investigations it seems to us, however, that the reports in literature of the easy transformation of bacteria into new strains with completely different properties must be taken with due caution, for such a transformation would inevitably involve fundamental changes in the enzyme-system of the cell.

As a substantial part of life processes, the enzymes are indispensable for the living cell and it is therefore of interest to study the relationship between life and enzyme action. We have made quantitative determinations with several bacterial strains to ascertain the influence upon the growth of bacteria of various factors,—including the temperature and the acidity of medium,—as well as the influence of these factors upon the enzymic activity of the bacteria in question (Virtanen, Wichmann and Lindström 1927). In general these investigations indicate that the range of the enzymic activity of bacterial cells is much wider than the range inside which growth can take place. Growth is naturally impossible under circumstances inhibiting the action of the life-supporting enzymes.

Our investigations on the heat-resistance of bacterial spores throw some light also upon the question of the actual factor causing the death of cells. Two speculative theories have been presented in an attempt to account for the exceptional heat-resistance of spores: the assumption that the resistance is attributable to a high insulating capacity of the cell-membrane, and the assumption that the chemical composition of the spore-contents—especially as regards proteins and inorganic salts—is to such an extent different from that of the vegetative cells as to result in a different effect of heat on the spore proteins. A simple calculation is, however, sufficient to show the absurdity of the former alternative, as the heat-insulating capacity of the cell-membrane would have to be about a million times higher than that of the air. The latter alternative, on the other hand, can only be demonstrated by experimentation. Together with L. Pulkki, (1933) I have determined the chemical composition of the vegetative cells and the spores of *Bacillus mycoides*, with the result that no actual differences were found to exist. The content of moisture, ash and protein was the same in both cases. Great differences were, however, found between the enzyme actions of spores and of vegetative cells. The enzymic activity of spores—especially that of the most important enzymes such as catalase and zymase—was nil, whereas the vegetative cells showed a powerful enzymic activity. The vital enzymes of the spores are obviously combined, by their active groupings, with the cell proteins in a way that renders them incapable of action and far more resistant toward heat than when in the active state. This would explain the resistance of spores towards heat. It may also be generally deduced from our findings that the *thermal* death of the cells is generally attributable to the destruction of enzymes and not to the heat coagulation of cell proteins, as is frequently stated to be the case. If, for some reason, the enzymes are preserved intact, the cell will survive the effects of heat.

The enzyme-protecting effect of proteins is also clearly manifested in the case of proteinase, secreted by the gelatin-liquefying bacteria (Virtanen and Tarnanen 1932a). The proteinase secreted by *B. fluorescens-liquefaciens* resists boiling for ten minutes with only a slight loss of activity, if the medium contains soluble proteins, whereas a complete destruction of the enzyme occurs in protein-free medium upon an exposure to 60° for a short time. Even in this case the resistance of the enzyme towards heat is to be attributed to a compound formed between the active enzyme groupings and protein bodies present in the medium, since the action of this proteinase is wholly inhibited, in a medium which contains proteins, at temperatures exceeding 60°, although the enzyme itself is thereby not destroyed and the velocity of the reaction should consequently increase with rising temperature. This applies also to bacterial lipases which are remarkably heat-resistant in media containing protein (Tammisto, 1933).

Owing to their ability to utilize a great number of different carbon compounds the bacteria provide excellent subject-matter for the study of the nutrition uptake of the cell, especially from the enzymatic point of view. The fact that certain bacteria are able to utilize complex organic compounds like cellulose may help us to gain insight into the important question, whether the bacteria are able, without preliminary hydrolysis, to utilize certain high-molecular compounds which are known to be decomposed through the action of hydrolases. According to Winogradsky, (1927, 1928, 1929) cellulose may be converted by certain bacteria into "oxycellulose," the oxidation of cellulose thus occurring without a preliminary hydrolysis to hexoses.

In this matter, our investigations have shown that the decomposition of cellulose is effected exclusively by microörganisms which produce the enzyme cellulase, i.e. are capable of hydrolyzing cellulose. The breakdown of cellulose is thus always preceded by an enzymic hydrolysis. In his dissertation Simola (1931) has shown that "oxycellulose" and similar substances mentioned in literature will also be formed from glucose and that they contain nitrogen. The humic acids that were produced by the aerobic cellulose-fermenting bacteria isolated by Simola, were also formed both from cellulose and from glucose.

In each investigated case it was shown that high-molecular insoluble compounds must undergo a preliminary hydrolysis before the cell can use them for its metabolism. The same holds true of soluble compounds with lower molecular weight, but capable of a further hydrolysis, as for instance the disaccharides. By following the velocity of the hydrolysis and fermentation of maltose by yeast, Willstätter *et al.*, (1921, 1922) deduced that maltose can also be decomposed by yeast directly, without a preliminary hydrolysis—a view opposed by v. Euler and Josephson (1922). It has also been generally assumed that bacteria may decompose lactose directly, since evidence has been lacking to show the production of lactase by lactose fermenters. We have, however, been able to show the presence of lactase in all lactose-fermenting bacteria investigated, and thus to prove the invalidity of the above assumption (Karström 1932). The same is true of the fermentation of maltose. Our work proved definitely that not a single one of the investigated bacterial strains could decompose a disaccharide if it did not produce the corresponding carbohydrase. From this, the general conclusion can be drawn that compounds decomposable by hydrolases must invariably undergo a hydrolysis before they can be utilized in the nutrition of the cell.

The ability of different bacterial strains to utilize as nutrients many different compounds facilitates greatly the solution of problems of this kind. The same is true as regards studies on the specificity of enzymes.

Some years ago Weidenhagen (1928, 1929) proposed a new hypothesis regarding the specificity of carbohydrases. He claimed that specific disaccharide-splitting enzymes,—like sucrase, maltase and lactase—do not exist. According to him, sucrose may be hydrolyzed by α-glucosidase and by a β-h-fructosidase, and maltose only by α-glucosidase. There exist, however, certain bacterial strains that hydrolyze maltose but not sucrose. Working with such a strain of *Bact. coli,* Karström (1930) proved beyond all doubt, that this strain was incapable of hydrolysing cane sugar although it hydrolysed maltose powerfully. The bacteria were used both as autolysates and as dry preparations. The much-discussed theory of Weidenhagen can thus not be valid, and we are still bound to accept the existence of specific disaccharide-splitting enzymes, like maltase, sucrase etc. (Virtanen, 1931; Myrbäck, 1931). This view is also corroborated by the findings of Pringsheim, et al. (1931) and of Kleiner and Tauber (1932).

It might *a priori* be conceived that the decomposition of high-molecular compounds, through the action of hydrolases, into a form suitable for metabolism, takes place in the medium, outside the cell. In such cases the cell would thus have to excrete the enzymes in question. An actual excretion by bacteria has, however, been held improbable, and the presence of enzymes in the nutrient solutions has been explained as due to autolysis. Together with J. Tarnanen (1932a) I have however shown that the gelatin-liquefying bacteria (even quite young cells) excrete practically quantitatively the enzyme proteinase, which hydrolyses proteins to polypeptides. The enzymes polypeptidase and dipeptidase are, on the other hand, not excreted into the medium but completely retained by the cell. The utilisation of proteins by the gelatin-liquefying bacteria is a very clear phenomenon: complex protein substances are first converted into soluble peptides, through the action of the secreted proteinase, outside the cell, whereas the further decomposition of peptides is effected by poly- and dipeptidases inside the cell.

It is more difficult to explain the utilisation of proteins by bacteria which do not liquefy gelatin. According to our determinations, even these bacteria contain both proteinase and peptidases but the proteinase is not excreted—a fact that accounts for the difference between the gelatin-liquefying and non-liquefying strains. However even the latter vigorously decompose complex proteins like casein and, since the proteins cannot permeate into the cell, it must be assumed that the proteinase-action takes place at the surface of the cell. We have paid particular attention to the proteolytic enzyme-systems of the important cheese-ripening bacteria, *Bact. casei* ε and *Streptococcus lactis*, and found them to be very similar in both cases. The proteinase-effect of *Bact. casei* ε is quantitatively much higher than that of *Str. lactis*; the polypeptidase-effect is about equal, whereas the dipeptidase-effect of *Str. lactis* is negligible. The pH- and temperature-curves of the said enzymes throw important light on the problems of cheese-ripening.

Proceeding to the true metabolism reactions, notably fermentations, and to the formation of proteins and other cell constituents, we encounter a variety of complex enzyme reactions, the true nature of which is in most cases still unrevealed. This is especially the case with synthetic reactions. We can only admire the synthetic powers of the cell, but are unable to explain the course of the reactions involved, on the basis of our present knowledge. The existence of separate enzymes catalyzing solely synthetic reactions is very doubtful. All processes involving enzymic synthesis have been shown to be simply enzymatic equilibrium reactions. The results obtained by using isolated enzymes are not sufficient to give us even the faintest picture of the synthetic activity of the living cell.

One of the central problems in the metabolism of the cell is the identity of the intermediate product, or products, of carbohydrate breakdown, from which the cell constituents are built up. Although our views on the fermentation of sugar have been greatly widened—thanks especially to the researches of Harden, v. Euler, Neuberg and their collaborators, carried out mainly with yeast—we know very little of the nature of the intermediate products utilized for the synthesis of the cell constituents. This problem is particularly complicated by the fact that the fundamental synthesis of the cell—the formation of amino acids—cannot be effected with killed cells or cell-free enzyme preparations. For instance, all our attempts to effect the breakdown or synthesis of alanine, using bacterial cells that were killed with different antiseptics, have utterly failed, though the deamination of alanine was easily effected by the strains used, in the absence of antiseptics.

The only case, in which the synthesis of an amino acid from a fermentation product of carbohydrates has been established with an isolated enzyme, is the synthesis of aspartic acid from fumaric acid. This reversible deamination was first discovered by Quastel and Woolf (1926) working with "resting" cells of *Bact. coli.* We have been able to isolate the corresponding enzyme, aspartase, from the cells of *B. fluorescens-liquefaciens* and to study the reaction more closely, using cell-free enzyme solutions (Virtanen and Tarnanen, 1932b). It has been shown that the action of aspartase is strictly specific, being limited exclusively to the system: l-aspartic acid $\rightleftarrows$ fumaric acid + NH_3.

The enzymatic synthesis of aspartic acid is theoretically very interesting since fumaric acid is formed from succinic acid through a simple enzymic reaction, succinic acid again being a common product in the fermentation of sugar.

Our investigations on bacterial fermentations may be briefly summarized as follows:

The *phosphorylation of sugar,* discovered by Harden (1905) in alcoholic fermentation, was shown to be a common reaction in different breakdown processes of sugar. This was demonstrated first in the case of lactic fermentation (Virtanen 1925b) and subsequently in the propionic fermentation (Virtanen, 1924, 1925a). Likewise it was shown that the presence of co-enzyme is indispensable for these fermentations. The identical nature of the earlier stages of different fermentations was thereby proved.

As phosphorylation occurs only with killed cells or cell-free extracts, it has been held even quite recently that this phenomenon is pathologic and does not occur normally (Nord, 1930). This opinion is based mainly on the fact that phosphate addition does not noticeably accelerate the fermentation by living yeast. With bacteria the case is different. Fermentations by lactic, propionic and coli-bacteria are increased by about 100 per cent through a phosphate addition (Virtanen, Wichmann and Lindström, 1927; Virtanen, 1931). Phosphorylation must, consequently, be a partial reaction of the fermentation processes caused by living organisms.

On the other hand our investigations (Virtanen and Tikka, 1930) with lactic bacteria show that the fermentation effected by dried cells in the presence of toluene differs considerably from that effected by live cultures. In the latter case sugar is almost quantitatively converted into lactic acid, whilst dried bacteria, in the presence of toluene, produce only about 50 per cent lactic acid, the remaining 50 per cent of sugar being changed through a hexose monophosphoric ester into stable phosphoric esters. This observation shows how the course of fermentation depends on the harmony existing between the different enzymes of the zymase-complex. The results obtained with killed cells must therefore not be applied offhand to normal fermentations. For the same reasons it is difficult to draw any conclusions respecting the construction of the actual intermediate phosphoric ester, from what we know of the isolated esters.

The *reactions following phosphorylation* are oxidations and reductions of different kinds. The exact nature of these processes has not been fully established as yet. In my opinion, the course of the fermentation of a simple sugar is best illustrated by our work on the decomposition of dihydroxyacetone by *Bact. coli* (Virtanen, Karström and Turpeine, 1929, 1930). We found that the first stage of the fermentation of dihydroxyacetone is obviously a phosphorylation, followed by a Cannizzaro reaction in which one molecule of dihydroxyacetone is reduced to glycerol and the other oxidised through glyceric acid to acetic and formic acids, thus:

$$(1)\qquad \underset{\text{Triose}}{2C_3H_6O_3} + H_2O = \underset{\text{Glycerol}}{C_3H_8O_3} + \underset{\text{Glyceric acid}}{C_3H_6O_4}$$

$$(2)\qquad \underset{\text{Glyceric acid}}{C_3H_6O_4} = \underset{\text{Acetic acid}}{CH_3COOH} + \underset{\text{Formic acid}}{HCOOH}$$

The frequent occurrence of formic acid among the end products of different fermentations is thus attributable to the decomposition of glyceric acid.

A comparison between the fermentations of glucose and dihydroxyacetone by the same strain of *Bact. coli* showed that glucose gave about 40 per cent of lactic acid but no glycerol. This means that the glucose molecule is not primarily disrupted into two triose molecules which would subsequently be dismuted further. In our opinion, glucose is first converted into methyl glyoxal (as assumed by Neuberg) which gives rise to lactic acid. I should attach particular importance to this observation especially since Nilsson (1929, 1930, 1932) has isolated phosphoglyceric acid from a fermentation by yeast in the presence of fluorides, and Embden (1933) from muscles; and these findings have served as a basis for various hypotheses regarding the mechanism of sugar breakdown. Nilsson's theory of the decomposition of hexose monophosphoric acid into glyceraldehyde phosphoric ester and some 3-carbon compound (possibly methyl glyoxal) is not out of harmony with our findings, whereas our results do not corroborate Embden's view of the disruption of hexose diphosphate into two molecules of phosphorylated trioses.

In most fermentations studied the decomposition of sugar obviously proceeds along the same lines up to the rupture of the 6-carbon chain into 3-carbon compounds. The subsequent reactions—oxidations, reductions, condensations, decarboxylations, etc.—are somewhat different in different cases and give rise to different end products.

The only known exception to the normal mechanism of fermentation is the formation of succinic acid from sugar. Succinic acid has been generally held to be formed from acetic acid through dehydrogenation, and Butkewitsch's work with molds (Butkewitsch and Fedoeroff, 1929, 1930) seems to support this view. In bacterial fermentations the case is decidedly different. In our; opinion (Virtanen, 1923, 1925, 1927; Virtanen and Simola, 1927 Virtanen and Karström, 1931) succinic acid is formed from sugar by a split of the hexose molecule into a 4- and a 2-carbon compound. Propionic fermentation furnishes ample evidence to support this theory. Live cultures produce some 10 to 20 per cent of succinic acid along with propionic and acetic acids and carbon dioxide. With dried bacteria in the presence of toluene the actual propionic fermentation does not proceed further than to the hexose monophosphoric acid stage (the ester has been isolated by us) whilst the production of succinic acid goes on undisturbed, the two different processes being thus distinctly separable from one another. In this respect, the highly important observation was made that no carbon dioxide is formed in the fermentation with dried bacteria. This proves conclusively that succinic acid does originate from sugar. The same result was arrived at with living bacteria, for in this case the fermentation gives rise to equivalent amounts of acetic acid and carbon dioxide.

I have been particularly interested in the formation of succinic acid for two reasons, first, since it is obviously a process substantially differing from the normal breakdown of sugar, and secondly, since succinic acid is very probably an important factor in the amino acid synthesis. Enzymic dehydrogenation of succinic acid results in the formation of fumaric acid which, through the action of the enzyme aspartase, takes up ammonia with the production of l-aspartic acid, as mentioned above. We might conceive that young, vigorously growing cells utilize succinic acid for the aspartic acid synthesis, whilst, for instance, in the absence of suitable nitrogenous nutrients the protein synthesis is much reduced and succinic acid collects in the medium. This assumption is in agreement with the observation made by us that *Bact. coli* produces much succinic acid in a medium containing very little ammonium salts, whereas only negligible traces of succinic acid are formed in the presence of great amounts of ammonium salts.

Our opinion of the formation of the important plant acids—malic, citric and aconitic acids—from succinic acid through fumaric acid, may be represented as follows: fumaric acid → malic acid → oxalacetic acid + acetaldehyde → citric acid → aconitic acid. It is true that the last-mentioned acids do not occur among the metabolic products of bacteria but they are frequently produced by moulds and higher plants. The central position of succinic acid in the metabolism of the cell is clearly seen from the above.

I wish to express my thanks to the Society of American Bacteriologists for their kind invitation to submit a paper on the subject of our investigations on bacterial enzymes and bacterial

fermentations. I regret that this paper deals one-sidedly with our own work while reference has unavoidably been omitted to much valuable research work with which other investigators, notably Americans, have during the last decades contributed to our knowledge of the various aspects of bacterial metabolism.

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PRESENT STATUS OF THE PROBLEM OF SUGAR FERMENTATION[1]

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Modern research on the alcoholic destruction of sugar began in 1897 with the discovery of E. Buchner that the process is of enzymatic nature. In 1905, Harden and Young found that the zymase of Buchner consisted of a thermolabile ferment fraction, which was later designated as "apo-zymase" by Neuberg and v. Euler, and of a thermostable ultrafiltrable factor, "co-zymase," the presence of which is necessary for the fermentation of sugar. Later it was recognized that phosphate was necessary for alcoholic fermentation. This circumstance led in 1910 to the drawing up of Harden's fermentation equation:

$$2C_6H_{12}O_6 + 2PO_4HR_2 = C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O + 2CO_2 + 2C_2H_5OH$$

It states, that one molecule of sugar always forms an ester and that a second molecule of sugar decomposes to ethyl alcohol and carbon dioxide. No consideration, however, was given to the mechanism of the formation of CO_2 and C_2H_5OH out of $C_6H_{12}O_6$, for neither alcohol nor carbonic acid is preformed in the sugar molecule in any form whatsoever. The first success in the search for intermediary products was Neuberg's discovery in 1910 that pyruvic acid is broken down into carbon dioxide and acetaldehyde according to the equation:

$$CH_3{\cdot}CO{\cdot}COOH = CO_2 + CH_3{\cdot}CHO$$

by all yeasts that ferment hexoses as well as by the enzyme preparations (carboxylase) prepared from the former.

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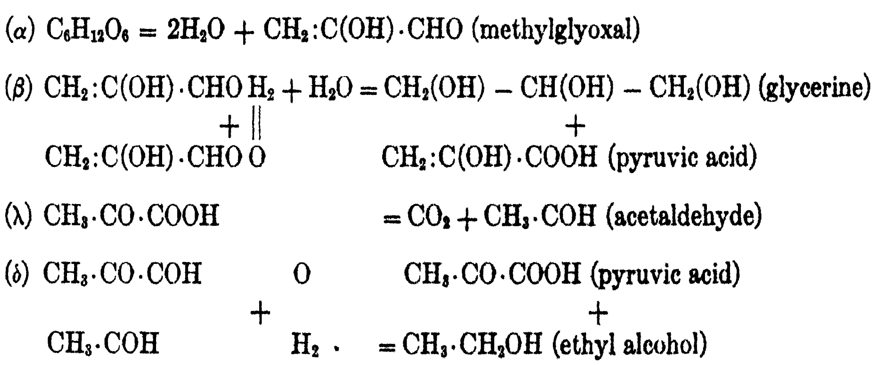
We have in pyruvic acid, which is fermented in the enolic form ($CH_2{:}COH\cdot COOH$) according to Neuberg and Weinmann (1928), therefore, a compound that directly produces one end-product, CO_2, of alcoholic fermentation by a simple biochemical splitting-out process which stands in contrast to the difficult decomposition of the α keto-acid, and in addition acetaldehyde which serves as a direct precursor of the second end-product. In 1913 Neuberg as well as Dakin and Dudley discovered the *biochemical transmutation of methyl glyoxal* (pyruvic acid aldehyde), i.e., its oxido-reductive *transformation into lactic acid.* In the same year Neuberg's fermentation scheme was formulated:



$$(\alpha)\ C_6H_{12}O_6 = 2H_2O + CH_2{:}C(OH)\cdot CHO \text{ (methylglyoxal)}$$

$$(\beta)\ \begin{array}{lll} CH_2{:}C(OH)\cdot CHO & H_2 + H_2O = & CH_2(OH) - CH(OH) - CH_2(OH) \text{ (glycerine)} \\ \qquad\qquad + & \| & \qquad + \\ CH_2{:}C(OH)\cdot CHO & O & CH_2{:}C(OH)\cdot COOH \text{ (pyruvic acid)} \end{array}$$

$$(\lambda)\ CH_3\cdot CO\cdot COOH \qquad = CO_2 + CH_3\cdot COH \text{ (acetaldehyde)}$$

$$(\delta)\ \begin{array}{lll} CH_3\cdot CO\cdot COH & O & CH_3\cdot CO\cdot COOH \text{ (pyruvic acid)} \\ \qquad\qquad + & & \qquad + \\ CH_3\cdot COH & H_2 & = CH_3\cdot CH_2OH \text{ (ethyl alcohol)} \end{array}$$

Up to the first publication cited the bases of the formulae were chiefly of a theoretical nature. Later, all of the important statements of the scheme were experimentally proved. The process begins with a reactive form of sugar, *am-hexose;*[2] that is, broken down into two molecules of a substance with three carbon atoms, which substance easily forms methyl-glyoxal by giving up water (α). This yields glycerine and pyruvic acid (β). Through the action of carboxylase the pyruvic acid decomposes to acetaldehyde and carbon dioxide (γ). Finally, out of methyl-glyoxal and acetaldehyde, through a mixed reaction, pyruvic acid along with ethyl alcohol is formed (δ). Methyl-glyoxal is always formed from the am-hexose, and CO_2 + acetaldehyde are continually produced from pyruvic acid. Accordingly, after the formation of but one molecule of glycerine—which is in fact a constant fermentation by-product—all the remaining reactions in the cycle can take place. The important point in this fermentation paradigm is the repeatedly occurring oxido-reductions, in the course of which the oxygen of water oxidizes the molecule, and the hydrogen reduces another molecule. The appearance of trioses was purposely not taken into consideration.[3] Should they ever be found to be intermediate products, their place in the scheme is an obvious one, as has been set forth in both of the monographs mentioned.

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[2] Alloiomorph.

The different stages in the breaking-down of hexoses by yeast were verified by appropriate experiments. The carboxylatic cleavage of pyruvic acid had been realised in 1910, as has been already mentioned. The separate intermediate products were successfully isolated in practice, partly by the fixation of an intermediate form by a fixing agent (*fixation procedure*), partly by the variation of the hydrogen ion concentration of the medium, and partly through alteration of the proportion of substrate to complex enzyme material, or to the activating-apparatus of the latter, making allowance for circumstances (*ferment-inhibiting procedure*).

Neuberg and Reinfurth in 1916 and 1918 established the intermediate nature of the aldehyde by the sulfite fixation procedure (*second type of fermentation*) through which up to 80 per cent of the theoretically possible amount of acetaldehyde was fixed in the form of a bisulfite compound. At the same time glycerine is formed by reduction equivalent to the product of oxidation, acetaldehyde, according to the equation of the second type of fermentation:

$$C_6H_{12}O_6 = CH_3 \cdot CHO + CO_2 + C_3H_8O_3$$

The same authors have established in special experiments that the pyruvate-sulfite-complex undergoes an easier carboxylatic cleavage than pyruvic acid itself. In 1920 Neuberg and Reinfurth brought about the fixation of the intermediately formed acetaldehyde also by means of Dimedon (dimethylhydroresorcinol), and later Neuberg, Kobel, and Tychowski (1927–28) as well as Klein and Fuchs (1929) used acid-hydrazids, which react slowly with hexose and at the same time are only slightly poisonous, as fixing agents for the acetaldehyde.

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[3] For this outline of the fermentation scheme see C. Neuberg, Monogr. Jena (1913), and in Oppenheimers Handb. d. Biochemie, vol. II, 2nd edition (1924), 461.

In an alkaline medium a change of the intermediately formed acetaldehyde to ethyl alcohol and acetic acid takes place (*third type of fermentation*, Neuberg and Hirsch, 1919). Glycerine is again formed as a reduction product equivalent to this biological formation of the oxidation product of acetaldehyde, according to the equation of the third type of fermentation:

$$2C_6H_{12}O_6 + H_2O = 2C_3H_8O_3 + 2CO_2 + C_2H_5OH + CH_3\cdot COOH$$

Neuberg and Kobel confirmed the cleavage of sugar into two compounds containing 3 carbon atoms only in 1928, when they attained an accumulation of methyl-glyoxal as a result of the action of the autolytic product of yeast on hexose-di-phosphoric acid salt and isolated this keto-aldehyde as bis-2,4-di-nitrophenylhydrazone. They designated this transformation:

$$C_6H_{12}O_6 = 2CH_3\cdot CO\cdot COH + 2H_2O$$

as the *fifth type of fermentation*. The methods were later considerably simplified and improved. The change of sugar to methylglyoxal is brought about by the action of component ferments of the complex apo-zymase, which effect a decomposition without the coöperation of co-zymase, and which remain active for years in dry yeast. The method which leads to the accumulation of methyl-glyoxal is also a *ferment-inhibiting procedure*. The keto-aldehyde is collected when apo-zymase that is free from, or poor in co-ferment attacks hexose-di-phosphate. Since these agents are abundantly present in apo-zymase, one can also proceed by using very small quantities of normal yeast in the presence of a plasmolytic substance such as, for example, toluol or brom-benzole and many other plasmolytic agents. The co-ferment is also sufficiently inactivated by that means—at least the keto-aldehyde formed remains, since co-ferment is necessary for its further assimilation. Auhagen and Neuberg have only lately demonstrated that the methyl-glyoxal formed as an intermediate is converted into lactic acid, in the presence of glutathion, the co-ferment of lactic acid fermentation. This *switching of the alcoholic destruction of sugar to lactic acid fermentation* is supported by the preceding appearance of methyl-glyoxal.

Since the co-ferment must also be put out of operation in order that the accumulation of 3-carbon-atom bodies may occur, it is natural that the realization of the fifth type of fermentation was not successful when normal hexose was used as a starting material. We know, that the processes of the "first attack" occur in the presence of co-ferment phosphate-esterification, and that the form of hexose capable of reacting, am-hexose, is first created thereby. One can proceed, therefore, only with such an *am-form* of sugar, as is presented by am-fructose-1,6-di-phosphoric acid, when the dividing in two of hexose without the assistance of co-ferment is aimed at. Methyl-glyoxal accumulated in the same way through the action of numerous other cells, of rarer yeasts, of fungi, of bacteria, and of tissues of higher plants and animals could also be isolated by Neuberg and Scheuer out of the reaction mixtures as a *dioxime* as well as in the form of angular *3-methylnaphthopyracine.*

Up to now methyl-glyoxal from the alcoholic destruction of sugar by yeast has been obtained with a yield of 85 per cent, and from the bacterial lactic acid fermentation up to 100 per cent of the theoretical.

The intermediate formation of pyruvic acid, which was provided for in the fermentation scheme drawn up by us, was made fully certain in 1929 by Neuberg and Kobel. Indeed a few earlier references on the presence of pyruvic acid in fermentation experiments exist. The identification itself was often indirectly accomplished, the yield was always inconsiderable, and indeed in most cases not to be distinguished from an oxidative formation. The pure anäerobic decay of hexose to pyruvic acid and glycerine was realised by Neuberg and Kobel to 100 per cent of the theoretical. The process corresponds to the equation:

$$C_6H_{12}O_6 = CH_3 \cdot CO \cdot COOH + CH_2OH \cdot CHOH \cdot CH_2OH,$$

and was designated by the authors as the *fourth type of fermentation.* Under particular conditions hexose-di-phosphate as well as free sugar was broken down into two C_3-bodies. With the use of hexose-di-phosphate as a substrate the experimental procedure was similar to that which permitted the isolation of methylglyoxal. It differs from the latter procedure only in that a little larger quantity of enzyme is required for the formation of pyruvic acid. The formation of pyruvic acid out of zymo-di-phosphate takes place very rapidly by the action of juice from macerated yeast or of fresh yeast, in the presence of toluol or other plasmolytic agents.

When the substrate does not consist of phosphorylated sugar, a displacement of the hydrogen ion concentration towards the alkaline side by addition of trimagnesiumphosphate, disodiumphosphate, or magnesium oxide, leads to a pH between 7 and 8 at the beginning. The pH at the end lies then between 5 and 7, while the pH at the termination of normal fermentation with living yeast is around 3.1. In yeast-juice experiments an introductory pH of about 6.9 is necessary for glycerine-pyruvic-acid cleavage. The possibility of production of pyruvate is present in that the action of carboxylase is inhibited with the H ion concentration displaced toward the alkaline side (Hägglund). The breaking down of the ester of hexose-di-phosphoric acid is connected with a dephosphorylation, and, on using free sugar as a substrate, first of all an esterification takes place and then, with the giving up of water, the formation of pyruvic acid occurs.

By the action of dried yeast on a mixture of glucose, acetaldehyde, hexose-di-phosphate and inorganic phosphate in the presence of sodium fluoride Nilsson observed in 1930 the formation of a phospho-glyceric acid that he considered identical to the glyceric-acid-mono-phosphoric acid, synthesized by Neuberg, Weinmann, and Vogt in 1928. Neuberg and Kobel simplified the biochemical preparation of phosphoglyceric acid by showing that neither dried yeast nor already formed hexose-di-phosphate was necessary for its production. They demonstrated, in addition, its formation out of sugars of very different sorts (hexoses, glyceraldehyde-phosphate, disaccharides). They also showed that the acetaldehyde can be replaced by other aldehydes, and that the acid of the 3-carbon-atom series mentioned is formed in the presence of toluol by action of maceration juices as well as of fresh yeasts, and established that it changes over to pyruvic acid just as hexose-diphosphoric acid does and under the same conditions. Finally, under conditions optimum for the decomposition of pyruvic acid by carboxylase, they accomplished the *fermentation of simple glyceric acid and also of its twofold phosphorylated ester.* If one now supposes that phospho-glyceric acid which is only obtainable under abnormal conditions—i.e., in the presence of a hydrogen acceptor and by fluoride poisoning—actually appears also in the course of normal decomposition, then it is easy to insert it in the Neuberg fermentation scheme mentioned at the beginning. It then appears as a phosphorylated precursor of pyruvic acid, which is fixed by the ionic poisoning of the medium. If desmolysis, the rupturing of the carbon chain, sets in on phosphorylated amhexose, then the phosphoglyceric acid + glycerine-phosphoric acid or phosphoglyceric acid + glycerine, as the case may be, are to be inserted before the pyruvic acid + glycerine, or making allowance for circumstances, logically before the end-product of the second and third type of fermentation.

After Fischer and Baer had synthesised in 1932 glyceraldehyde-3-mono-phosphoric acid and Smythe and Gerischer had established that the dextrorotatory components ferment with yeast with about the same velocity as zymo-hexoses, the possibility that the glyceraldehyde-phosphoric acid forms an intermediate product in the alcoholic decomposition of sugar was taken into consideration. Neuberg and Kobel recently showed that in the presence of sodium fluoride its transmutation by yeast takes place without splitting-out phosphate and yields phospho-glyceric acid. All of these findings, however, are no proof of the real nature of this compound as an intermediate product. Let the cases of oxalacetic acid and of d-Mannononose be recalled, which in spite of fermentability are not to be considered as intermediate forms. As long as one has not succeeded in isolating phosphoglyceraldehyde out of ferment mixtures, perhaps calorimetric measurements may throw light on the possibility of an intermediate formation of glyceraldehyde-phosphoric acid. For the free glyceraldehyde, which it is true could only be used in dimolecular form for the determination of the heat of combustion, no basis is presented, to warrant the conception of its rôle as an intermediate product according to the energetic measurements of Neuberg, Hofmann and Kobel. The isomer, dihydroxyacetone, is excluded as such for energetic reasons, as well as accordng to the biochemical findings of Neuberg and Kobel and of Iwasaki who established in 1928 that in the fermentation of dihydroxyacetone first of all hexose-phosphoric acid ester forms and the decomposition occurs only after condensation to hexose sets in.

The existence of a triose-phosphoric acid ester was already taken into consideration years ago. In 1907 Iwanoff declared that the phosphoric acid ester isolated by him by fermentation contained triose-mono-phosphoric acid. In the same year Wohl developed the conception that glyceraldehyde appears as the first decomposition product of sugar by means of an aldol-depolymerisation. With loss of water it then changes over to methylglyoxal. In 1927 Kluyver and Struyk considered a decomposition of phosphorylated sugar, namely of hexose-mono-phosphate to glyceraldehyde and glyceraldehyde-phosphoric acid, for which process they supposed a phosphorylation in the 2-position. They strengthened this hypothesis with the circumstance that in distillation of the fermentation mixture with sulfuric acid according to the method of Neuberg, Färber, Levite and Schwenk, methyl glyoxal can be demonstrated to a small extent. As a matter of fact Neuberg and Kobel were able to determine with certainty the condition required for the production of triose-phosphate. It was only necessary to diminish the quantity of enzyme still more than is necessary for the production of methyl glyoxal. They then obtained with the same agents (i.e., yeasts, lactic acid bacilli and parts of green plants) large quantities—30 per cent and more—of esterified triose. Nilsson supposes that the decomposition of a hexose-mono-phosphoric acid commences with the equilibrium between a 6-position phosphorylated form of enolic character (see Neuberg, 1913) and the two stable esters of hexose-mono-phosphoric acid, fructose-6-phosphoric acid (Neuberg's ester) and glucose-6-phosphoric acid (Robison's ester) respectively, and that with the assistance of co-enzyme it is broken down to glyceraldehyde-3-phosphoric acid according to the equation:

$$\begin{aligned} \mathrm{CHOH} &= \mathrm{C(OH)} - \mathrm{CHOH} - \mathrm{CHOH} - \mathrm{CHOH} - \mathrm{CH_2O \cdot PO_3H_2} \\ &= \mathrm{C_3H_6O_2} + \mathrm{CHO} - \mathrm{CHOH} - \mathrm{CH_2O \cdot PO_2H_2}. \end{aligned}$$

None of these three substances was isolated. The support for this scheme was only the finding of the author that in the presence of fluoride, which according to the investigation of Meyerhof (1927) and Lipmann (1928) inhibits fermentation and prevents the break-down of phosphoric acid ester, as well as in the presence at the same time of acetaldehyde and hexose-di-phosphate, a substance is formed which he declared to be identical with phosphoglyceric acid, Nilsson has established that in the presence of the ionic poisoning phenomenon mentioned acetaldehyde is reduced to alcohol and he thence concludes that it is a mixed reaction between acetaldehyde and glyceraldehyde-phosphoric acid to ethanol and phosphoglyceric acid. According to this view, however, glyceric acid is no normal intermediate product of desmolyses, but a fixation product like, for example, the acetaldehyde in the fixation process which makes use of sulfite. He accepts glyceraldehyde as a real intermediate product (see also Embden and Meyerhof and associates). Normally this is thought by Lebedew to arise from hexose-di-phosphate and to be condensed again to the latter, while the phosphorus-free compound $C_3H_6O_3$, the empirical composition of which corresponds to that of a hydrate of methyl glyoxal, is decomposed in known manner to alcohol and carbon-dioxide. According to this interpretation the fermentation equation of Harden would be fulfilled, which equation provides for a parallelism between the esterification to hexose-di-phosphate and the evolution of CO_2. Support for the intermediate appearance of glyceraldehyde-phosphoric acid and its condensation to hexose-di-phosphate is seen by the Swedish author in the fact that galactose in spite of its difference in structure produces the same hexose-di-phosphoric acid as the zymohexoses properly speaking. This finding does not, however, permit of its being accepted as a fully valid argument in as much as we know since the investigations of Neuberg and Leibowitz (1927) as well as Veibel (1931) that the diverse sugar esters of phosphoric acid change by a biochemical enolization extraordinarily easily from one to the other in the fermentation mixture, and that several of the esters are always simultaneously present. In addition, the well-known findings of Nef (1914) indicate that a transition from the galactose series to the glucose series also may take place chemically. In the triose-phosphates the energetic relations may be differently arranged than in the free triose, consequently, the possibility may exist that methyl-glyoxal has a precursor in phosphotriose, just as pyruvic acid has in phosphoglyceric acid. New findings suggest, it is true, that genuine glyceraldehyde-3-phosphoric acid does not at all produce methyl-glyoxal under conditions under which we isolated methyl-glyoxal in large quantities from hexose-di-phosphate with practically all types of cells. Indeed, phosphoglyceraldehyde gives rise to methylglyoxal upon heating, just as the free trioses do (Spoehr, Cameron, Neuberg and Collatz), when it is boiled in the presence of hydrochloric 2,4-di-nitrophenylhydrazine. With our experimental procedure alone—precipitation in the cold with the reagent and retention for one or two hours at room temperature—only traces of methylglyoxal-bis-hydrazone are formed at the most from glyceraldehyde-3-phosphoric acid. Hydrazones containing phosphorus, or free from phosphorus, making allowance for circumstances, are formed that are not derivatives of methyl glyoxal. Also under the influence of the enzymatic component complex of yeast, designated by us provisionally glycolase, phosphoglyceraldehyde gives rise to no methyl-glyoxal. The methyl-glyoxal we obtained biochemically from hexose-di-phosphate is not merely an artificial product formed from glyceraldehyde-phosphoric acid during the isolating procedure. The finding of Auhagen and Neuberg referred to also supports this view. The question must remain open, as to whether the course taken during the decomposition proceeds at all beyond phosphoglyceraldehyde. The phosphoglyceric acid formation observed under the effect of fluoride can not be applied in this respect without consideration. Attention is indeed to be paid, however, to the isomer di-hydroxy-acetone-phosphoric acid, since combined triose has been demonstrated with certainty to be among the products of glycolytic reaction (see foregoing pages). The formation of phosphoglyceric acid out of phosphorylated di-hydroxy-acetone would then assume a biological isomerisation, such as has been observed by Neuberg and associates in the case of the transformation of fructose-phosphate into glucose-phosphate. Di-hydroxy-acetone-phosphoric acid might then become methyl-glyoxal, analogous to the formation of pyruvic acid from glyceric-phosphoric-acid, and could be inserted in the fermentation scheme as a veritable intermediate product.

SOME PHYSIOLOGICAL CHARACTERISTICS OF THE PROPIONIC-ACID BACTERIA[1]

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A comprehensive review of the literature pertaining to the production of propionic acid as the chief product in the fermentation of many organic compounds by specific microörganisms was made by Van Niel in 1928. He agreed with Orla-Jensen (1909), who, stressing the dissimilatory conversions of this group of bacteria rather than their morphology, raised them to the generic rank Propionibacterium. In a comparative study of 30 cultures, which he isolated, Van Niel recognized seven species described by previous investigators and one new species. On the basis of morphological, cultural and fermentative differences these eight species were placed in the genus Propionibacterium (Orla-Jensen 1909) and designated as follows:

Propionibacterium Freudenreichii
Propionibacterium Jensenii
Propionibacterium Peterssonii
Propionibacterium Shermanii
Propionibacterium pentosaceum
Propionibacterium rubrum
Propionibacterium Thönii
Propionibacterium technicum

Werkman and Kendall (1931) studied the physiological behavior of 32 cultures of the propionic acid bacteria. They recognized the eight species of Van Niel and added one new species, *Propionibacterium raffinosaceum*.

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[1] Part of a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the Graduate School of the University of Wisconsin.

Hitchner (1932) made a comparative study of 16 cultures of the propionic-acid bacteria, which he isolated from cheese, milk, soil, and silage, together with six cultures obtained from other investigators. In this group six species of Van Niel were recognized and two new species, *Propionibacterium zeae* and *Propionibacterium arabinosum*, were suggested. Recently Werkman and Brown (1933) have shown that *Propionibacterium zeae* exhibits a serological specificity unique among the propionic-acid bacteria.

In conducting the investigation cited above (Hitchner, 1932), certain physiological characteristics of the propionic-acid bacteria were observed, which indicate that this group of microörganisms are somewhat unique in their metabolic processes. A presentation of these observations is given in the subsequent pages.

The sources and classification of the cultures employed are as follows:

Propionibacterium Freudenreichii—No. 33, author.
Propionibacterium Shermanii—Nos. 46 and 49, author; No. 31, Dr. Sherman, Ithaca, N. Y.
Propionibacterium Jensenii—No. 54, author.
Propionibacterium Thönii—No. 52, author.
Propionibacterium pentosaceum—No. 11, Dr. Van Niel, Holland; Nos. 57 and 58 differ from No. 11 only in their inability to ferment xylose and are tenatively placed in this species.
Propionibacterium arabinosum—Nos. 59, 60, 61, 62, and 63, author.
Propionibacterium zeae—No. 56, author.

PRODUCTION OF CATALASE BY REPRESENTATIVES OF THE GENUS PROPIONIBACTERIUM

Orla-Jensen (1907) reported that strains of *Bac. acidi-propionici* failed to decompose hydrogen peroxide, although *Bact. acidi-propionici a* caused a rapid evolution of gas when tested with this compound. Sherman (1921) (1926) emphasized the ability to produce large amounts of catalase as an outstanding characteristic of *Bact. acidi-propionici d.* He (1919) had earlier shown that oxygen was evolved when samples of Swiss cheese were added to a neutral solution of hydrogen peroxide. Since that time the ability to decompose hydrogen peroxide has been considered an important characteristic in the identification of representatives of this group.

In the isolation of our cultures, marked variations were observed in the rate at which the hydrogen peroxide applied to the colonies was decomposed. With most cultures the evolution of oxygen was quite rapid, but with four cultures a few bubbles of gas appeared only when the peroxide had been in contact for some minutes. So definite was the variation that the cultures were first considered as catalase negative. These results warranted a comparative quantitative study of the ability of certain cultures to decompose hydrogen peroxide for which a modification of the method employed by Virtanen and Karström, (1925) in a study of the catalase content of several species of bacteria seemed applicable.

Tubes containing 10 cc. of sodium-lactate broth were inoculated with a 6-mm. loop of three-day-old broth cultures of the respective strains, which—to insure vigorous growth—had been carried through several successive transfers in the same media. After 10 days incubation the tubes were thoroughly shaken, 5 cc. transferred to a 50-cc. volumetric flask and diluted to the mark with M/150 phosphate solution, pH 6.5. An aliquot, the amount depending upon the catalase content as determined by a preliminary test, was transferred to a 100-cc. Erlenmeyer flask, 5 cc. of approximately 0.1 N solution of neutral hydrogen peroxide added, and the mixture placed immediately in an ice-bath. After a definite time interval, again depending upon the activity of the culture, the flask was removed, the contents acidified with dilute sulphuric acid, and the residual hydrogen peroxide estimated by titration with 0.025 N potassium permanganate. To compensate for any decomposition of the hydrogen peroxide by the organic matter in the solution, a similar test with a heated culture was run as a check. Although some variation was noted when repeated tests were made on a culture, in general, the same proportional variation was observed among the various strains. Some typical results are given in table 1. For comparative purposes the data have been computed for the amount of 0.1 N hydrogen peroxide decomposed by 1.0 cc. of the various cultures.

As a further test of the variability of this property, the rate of destruction of the peroxide was determined by measuring the rise of agar plugs in sterilized glass tubing (60 by 0.8 cm.) containing a mixture of 5 cc. of a ten-day-old broth culture and 5 cc. of 1-per-cent hydrogen peroxide. The same general relationship as that noted in table 1 was observed. Those cultures which rapidly decomposed the hydrogen peroxide in the previous test caused a rapid rise of the plugs; in fact, so rapid was the evolution of gas that the plugs were blown from the tube almost instantly. Cultures 11, 57, 58, 60 and 61, however, produced a rise of less than 1 cm. in four days. A culture of *Str. lactis*, treated in a similar manner, failed to show any evidence of catalase activity in the same time.

TABLE 1

Decomposition of hydrogen peroxide by certain cultures of the propionic-acid bacteria

CULTURES	REACTION TIME	0.1 N H_2O_2 DECOMPOSED COMPUTED FOR 1.0 CC CULTURE
	minutes	*cc*
60	30	0.05
57	30	0.09
61	30	0.14
58	30	0.15
11	10	0.42
52	15	2.5
33	15	19.2
46	15	31.5
54	10	41.5
56	10	43.0
63	5	46.7

These results indicate that the variation in catalase content is an inherent property of individual cultures and not associated with any set of physiological characteristics. Karström (1930) in his studies with the members of the colon group, concluded that catalase should be considered a constitutive enzyme rather than one developed through adaptation. Virtanen (1931) in a study of the catalase content of aerobic and anaerobic bacteria found that many aerobes produce less catalase than the propionic-acid bacteria which are sensitive to oxygen. These last-mentioned bacteria were the richest in catalase of all microörganisms studied by him. Since he offered quantitative data for only one strain of the propionic-acid bacteria, it is possible that, were a larger number of strains investigated, a variation in catalase content similar to that noted by us would have been found.

In view of our results, which show that certain representatives of the propionic-acid bacteria decompose hydrogen peroxide only to a slight extent, it would appear that the general characterization of this group of microörganisms as vigorous producers of catalase should be modified.

FERMENTATION OF SUCCINATES

Von Freudenreich and Orla-Jensen (1906) reported that the three strains of propionic-acid bacteria, which they studied, failed to ferment succinates. Similar results were obtained by Virtanen (1923) with a strain which he believed identical with *Bact. acidi-propionici, var fuscum* of Thöni and Allemann. However, Shaw and Sherman (1923) emphasize the ability of *Bact. acidi-propionici d* to produce propionic and acetic acid in a solution containing peptone and succinates. They further suggest that succinic acid may be the source of some of the propionic acid and acetic acid found in Emmenthal cheese, since it has been shown to be present in cheese at the time the eyes are forming.

Because of the rather limited data relative to the fermentation of succinic acid by the propionic-acid bacteria, it seemed desirable to test our cultures for this property. Flasks containing 400 cc. of a medium composed of 2 per cent peptone and approximately 1 per cent of succinic acid were inoculated with a 5 per cent inocula of cultures 57, 58, and 62, isolated by us, and of culture 31 from Sherman. The succinic acid was neutralized with the calculated amount of strong sodium hydroxide before addition to the peptone broth. Another set of flasks containing only peptone-solution were similarly inoculated as checks. The flasks were incubated at 30°C. for forty-seven days, 100 cc. portions removed, acidified, and the volatile acids determined following distillation with steam. The increase in volatile acid when succinic acid was added to the peptone broth ranged from 1.9 to 4.9 cc. 0.1 N acid with the various cultures. Similar results were obtained when cultures 57, 58, 46, 49, 59, and 60 were grown in a medium containing yeast water, 1 per cent peptone, and approximately 1 per cent succinic acid.

The slight differences in the amount of volatile acids formed in the presence of the succinic acid are no greater than are often found in duplicate cultures in the same media. It would appear that under the conditions of these experiments all cultures failed to ferment succinates with the formation of volatile acids.

The following experiment would seem to indicate that in a fermenting glucose solution the fate of the succinates is somewhat different. Flasks containing 1 per cent glucose and 0.5 per cent succinic acid in yeast-water peptone-broth were inoculated with cultures 49 and 58 used in the preceding experiment and culture 11 from Van Niel. These were incubated for fifty-two days at 30°C. Parallel cultures in glucose yeast-water peptone-broth were included as controls. At the termination of the experiment the total volatile acids and the residual sugars were determined. With each of the cultures the percentage of sugar fermented (94.5 to 96.5 per cent) was the same in the presence of succinic acid as in the controls. In the glucose broth cultures 11, 49, and 58 produced 109.2, 102.1 and 112.1 cc. respectively of 0.1 N volatile acid in 100 cc. of culture. However, an increase in volatile acids, which ranged from 18.9 cc. 0.1 N acid in 100 cc. of culture with no. 11 to 74.5 cc. 0.1 N acid with no. 49, was found when succinic acid was added to the glucose yeast-water peptone-broth. No significant increase in volatile acids (1.3 cc.) was noted with no. 58.

Succinic acid has been detected in varying amounts in the propionic-acid fermentation of glucose. That it occurs as an intermediate stage in the fermentation is questioned by Van Niel. While the data in this observation are insufficient to justify any conclusions, the fact that succinic acid is fermented in the presence of glucose and not attacked in its absence suggests that with some strains, at least, it may be the origin of a part of the volatile products in the propionic-acid fermentation of glucose. It is possible that in the complex mechanism certain conditions favorable to the dissimilation of the succinic acid are developed which are not found when it alone is present. This observation suggests an interesting line of study for future work.

SUMMARY AND CONCLUSIONS

Quantitiative measurements of the hydrogen peroxide decomposed in a definite period by representatives of the genus Propionibacterium show striking variations in the catalase content of the cultures. From these observations it would appear that the general characterization of the propionic-acid bacteria as vigorous producers of catalase should be modified and that less emphasis should be placed upon this property as a diagnostic test.

Succinic acid is not utilized by any of the cultures when it is present as the sole source of carbon. However, data are submitted which show that with some strains volatile acids may be produced from succinates in the presence of a fermentable carbohydrate such as glucose.

The author wishes to express his appreciation to Dr. E. B. Fred, under whose general supervision the investigation was conducted, and to Dr. W. H. Peterson for helpful suggestions and criticisms.

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CAPSULES IN YOUNG CULTURES OF STREPTOCOCCUS HEMOLYTICUS

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During the past year we have been occupied in comparing, from various points of view, young and old cultures of the same strains of *Streptococcus hemolyticus*. The considerations which suggested such a comparison were the observed resistance of young organisms to phagocytosis, and the presence of a stainable capsular material in very young cultures. It is probable that this material differs from the conventional capsule described for eighteen- to twenty-four-hour cultures of the *Str. epidemicus* variety (Davis, 1912; Davis and Rosenow, 1912) of hemolytic streptococcus, since, unlike that variety it cannot be demonstrated by our technique in cultures older than six to eight hours, and cannot be stained by the usual methods.

EXPERIMENTAL

In order to obtain sufficient organisms for study in a very young culture, a large inoculum was used. Although the capsule can be shown on blood agar, in serum broth, in saline suspensions of organisms grown in serum broth, and, with difficulty, in plain infusion broth, it is most clearly observed when the organisms are grown in defibrinated fresh blood. Rabbit blood (2.0 cc.) was inoculated with 0.2 cc. of a twelve-hour 20 per cent horse serum infusion broth culture, and incubated at 37°C. Samples of the culture were examined at fifteen-minute intervals during the course of five hours in the following manner:

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A small drop was placed on a slide and spread out with another slide. After drying without heat, Wright's stain[2] was applied for one minute. This was then diluted with an equal amount of freshly distilled water and allowed to remain for three to five minutes. The film was then washed lightly in distilled water and allowed to dry without blotting. The best staining was found to occur at the extreme edges of the smear. The development of the capsules was observed as follows.

After the first fifteen minutes, no change in the morphology of the organism was seen. In one-half to one hour the structure shown in figure 1 appeared. Around each of the blue-staining cocci or chains, a pink granulated reticulum was seen. Occasionally this material was dense and compact. This appearance persisted for two to two and one-half hours, but by the time the culture was three or four hours old the capsule had degenerated to a ragged pink shred, or was entirely gone. The objection might be raised that by the use of so large an inoculum, the capsulated phase occurs within a much shorter period than would be the case were a conventional inoculum used, and that such

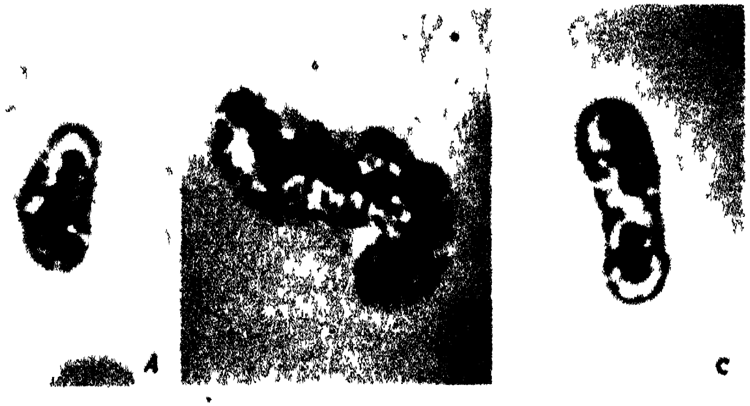

FIG. 1. STREPTOCOCCUS S23. ONE AND ONE-HALF-HOUR CULTURES, WRIGHT'S STAIN

A. Human blood culture 1500 ×
B. Rabbit serum culture 2000 ×
C. Twenty per cent horse serum broth culture 1800 ×

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[2] Three-tenths gram of the dry powder dissolved in 100 cc. of acetone-free absolute methyl alcohol, filtered, and allowed to stand twenty-four hours.

capsules would not differ from those hitherto described in twenty-four or forty-eight hour cultures. However, when a one-loop inoculum has been used, these capsules, characteristic of the young organisms, have never been observed in cultures older than six to eight hours.

To stain the capsules from serum broth, a loopful of the culture was mixed with a loopful of serum or defibrinated blood before spreading. Blood was found to be more suitable since it prevented the film from washing off as the stain was diluted. It was observed that after heating such a serum broth culture to 56°C. for forty-five minutes, capsules could no longer be seen. To demonstrate the capsules on solid medium, the growth was emulsified in a small drop of blood or serum on a slide, spread out, and stained as described above. In five hours there was usually enough growth on blood agar to make a satisfactory smear, and at this time capsules were present, although not as regularly as in the blood cultures. They began to disappear after eight to twelve hours on this medium, and have never been seen in twenty-four hour colonies except in the large isolated glassy, "pseudo-gloss" colonies found in strain S23 of Todd and Lancefield (1928).

Forty-nine throat strains of *Streptococcus hemolyticus* (including 28 from the House of the Good Samaritan in Boston, and 18 from the children's wards in the Virchow Hospital in Berlin) have been examined for this early capsulation, in addition to strains NY5, Aronson, and S23 of Lancefield. Of these, 15 showed it markedly, 4 to a less extent, and 30 not at all. The nineteen strains which exhibited this capacity for early capsulation showed, with one exception, uniform growth after ten hours' cultivation in 20 per cent horse serum broth. In a few of the strains there was a fine granulation, although this had not settled out. In contrast, the thirty non-capsulated strains, also with one exception, gave a very granular growth which settled out leaving a practically clear supernatant medium. That the presence of this capsular material confers stability on suspensions of streptococci in serum broth, was also demonstrated by the relative sedimentation rates in the centrifuge, of a young capsulated culture of S23, and an older uncapsulated culture of the same strain. The latter was easily sedimented by ten to fifteen minutes centrifugalization at 1800 r.p.m., while the former required thirty to forty minutes and was then not entirely cleared. By the colorimetric method, there was no difference in the pH of the two cultures.

The "matt" colony form of Todd (1928) could not be correlated with capsulation, since in only about one-half of the "matt' strains could capsules be demonstrated. Although the number of entirely "glossy" strains (7) was too small to be significant, none of them was capsulated. In the classification of Loewenthal (1932–3), all of the "M" or mucous colonies showed the capsular material, but so also did many strains which were not mucous in character.

One of the strains, S23 of Lancefield, was selected for study in an attempt to demonstrate further differences in the behavior of organisms in the capsulated and uncapsulated phases. This strain was very well capsulated, and it was shown that a one and one-half hour serum-broth-culture centrifugate occupied approximately three times the bulk of an otherwise comparable four-hour culture centrifugate, although there were many more organisms in the latter. A similar comparison of 2 one and one-half hour serum broth cultures, one of which had been heated to 56°C. for forty-five minutes (after which no capsules are demonstrable) showed that the sediment from the heated culture occupied about one-fourth that of the unheated culture sediment. It seems reasonable to assume that loss or alteration of capsular material plays some part in this decrease in bulk, and that the appearance of the stained preparations described above is probably not due to artefact.

Suspensions of young and old organisms were compared in regard to their stability in varying concentrations of NaCl.

A sixteen-hour and a one and one-half-hour 20 per cent horse serum broth culture were used. The sixteen-hour culture was prepared in the conventional manner, i.e., 250 cc. of 20 per cent horse serum medium were inoculated with a drop of a twelve-hour culture and incubated sixteen hours at 37°C. The one and one-half-hour culture was made by adding 25 cc. of a twelve-hour 20 per cent horse serum broth culture to 225 cc. of fresh horse serum medium (which had previously been warmed to 37°C.) and incubating for one and one-half hours at 37°C. These two cultures were iced, centrifugalized rapidly and the sedimented organisms taken up in 10 cc. of iced distilled water. Iced distilled water was then added to the suspension of old organisms until its turbidity was equal to that of the young suspension. The two suspensions showed the same pH by the colorimetric method. They were distributed in 0.5 cc. amounts in Wassermann tubes and 0.5 cc. of varying dilutions of NaCl were added so that a series of final NaCl dilutions ranging from 0.2 to 1.2 per cent at 0 2 per cent intervals was obtained. The agglutination was read after twenty-four hours in the ice-box The control tubes containing equal amounts of distilled water suspension and distilled water showed no agglutination with either the young or old organisms.

The young organism suspension was agglutinated at a NaCl concentration of 0.2 per cent while the old organisms were not agglutinated until a concentration of 0.8 per cent had been reached, and then the clumping was not as complete as in the case of the young organisms. These results were unexpected, considering the greater stability of the young phase in serum broth.

It is a common observation that young streptococci are more resistant to phagocytosis than old ones, and it was found that this loss of resistance was coincident with the loss or partial degeneration of the capsule.

Defibrinated fresh human blood (C. V. S.) was used in studying the phagocytosis. One-fourth cubic centimeter was placed in a small Pyrex test tube and after the addition of one standard drop of an appropriately diluted culture, sealed in a gas-oxygen flame, and slowly rotated end-over-end at 37°C. for twenty minutes. Blood smears were made from this as usual, stained with Wright's stain, and the organisms in 50 leukocytes counted. The per cent of leukocytes participating in the reaction was also determined. The organisms (S23) were grown in 20 per cent fresh horse serum broth for varying periods of time, and one standard drop used for each 0.25 cc. of blood. Cultures older than one and one-half hours were diluted with fresh broth until their turbidity was slightly less than that of the youngest culture. The supernatant from a twelve-hour culture was found to have no stimulating effect on the phagocytosis of the young form. In this system, organisms one to one and one-half hours old were taken up practically not at all, usually less than 10 cocci in 50 leukocytes. The same cultures heated to 56°C. for forty-five minutes usually showed about 600 cocci, occasionally as many as 1000 cocci per 50 leukocytes. After two hours growth, at which time the capsulated phase has approached its end, phagocytosis was already marked, usually 100 to 150 organisms in 50 cells, and at four hours this anti-phagocytic effect was entirely absent, phagocytosis having reached the same level as that observed in a twelve-hour culture, namely an average of 200 to 300 organisms in 50 leucocytes. The same observations were made with another blood (H. K. W.), and with dog blood. None of these three bloods showed any bactericidal effect against young or old organisms, by the technique of Todd (1927). It is of interest to note that during this work, the donor of blood C. V. S. contracted a hemolytic streptococcus sore throat, in which the infecting organism was proved to be S23. Subsequently the young capsulated organisms of S23 were vigorously phagocyted in this blood, and its bactericidal effect was markedly increased. The test organism had not altered, as it behaved as before in blood H. K. W., and was still fully virulent for mice.

The addition of homologous rabbit antiserum neutralized the anti-phagocytic property of the capsulated form. It is probable that this effect was type specific since the absorption of the antiserum with an heterologous hemolytic streptococcus (Lancefield, 1928) failed to remove its opsonizing property for the capsulated organism.

No difference in virulence between the capsulated and non-capsulated phase either by mouse inoculation, or by bactericidal tests in whole blood by the technique of Todd (1927), could be shown. This is not surprising when one considers the promptness with which capsulation of an old culture occurs after its introduction into a fresh medium. Extraction of the two phases in distilled water, in saline at 56°C., in N/20 HCl at 100°C. as described by Lancefield (1928), and by freezing and thawing, was carried out. No significant differences in the precipitinogen and complement-fixing titres of these young and old extracts could be observed when tested with either homologous antiserum, or with homologous antiserum absorbed with a heterologous hemolytic streptococcus. The sera of rabbits immunized with the living capsulated phase did not show a higher bactericidal titre than the sera of rabbits immunized with non-capsulated heat-killed organisms.

SUMMARY

Evidence is presented from which it seems reasonable to conclude that, in certain strains of *Streptococcus hemolyticus*, the young organisms differ structurally from the older organisms, and that this difference may influence the virulence of the strain.

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BACTERIOLOGICAL CHANGES IN ACIDOPHILUS MILK AT ROOM AND ICE-BOX TEMPERATURES

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What is the most desirable temperature at which to store acidophilus milk? The answer to this question depends upon one's point of view and is conditioned by a variety of conflicting considerations. It is generally agreed that acidophilus therapy depends in the last analysis upon the ingestion of a large number of viable *L. acidophilus*. In the treatment of chronic constipation or diarrhea we have recommended a daily dosage of one quart of acidophilus milk containing 200 billion viable organisms (Kopeloff, 1926). Whether or not such a requirement can be fulfilled when dealing with acidophilus milk on a commercial scale is a problem to be solved by the manufacturer. He must take into consideration: the palatability of his product, the contaminants therein and the time elapsing before use. Examination of commercial products has revealed a failure to meet specifications in many instances and only emphasizes the difficulties inherent in the problem (Kopeloff, Cohen, and Beerman, 1928).

In a previous report on some temperature studies of acidophilus milk it was shown that after three days' storage in the ice-box 90 per cent of the *L. acidophilus* organisms had been killed (Kopeloff, 1926). Comparing this with results previously published by Kopeloff and Cheney (1922) in which storage at room temperature (25° to 30°C.) for three days caused a decrease of 75 per cent we concluded that acidophilus milk of approximately 1 per cent acidity[1] would retain more viable organisms if stored at room temperature than in the ice-box. Reichart and Davis

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[1] In this and the previous experiment reported we neglected to publish the titratable acidity of our milk.

(1928) have published corroborative evidence but Black (1931) arrived at diametrically opposed conclusions. His experiments are open to criticism in that they are based on counts from single flasks and obvious inconsistencies remain unexplained. Similar comment might be made on a subsequent paper by Black and Harris (1931) dealing more particularly with the effects of acidity. Kulp (1931) has indicated that acidophilus milks of lower acidity maintained their viability to a greater extent than did milks of higher acidity. Prouty and Bendizen (1932) also claimed that acidophilus milk of high count and an acidity not exceeding 1 per cent might be effectively stored at low temperatures for a week.

There is one phase of the general problem which has received scant attention, namely, the rôles played by the rough and smooth variants of *L. acidophilus.* Most acidophilus milks are mixtures of rough and smooth, to say nothing of intermediate types. Since it is conceivable that rough and smooth types of *L. acidophilus* might react differently under the same temperature conditions, an experiment was planned to investigate this point, as well as to consider the influence of initial acidity on viability at room and ice-box temperatures. In the present study therefore we have employed two rough strains (R) yielding 100 per cent rough colonies with minute centers. One strain represented the progeny of a M. C. Kahn single cell isolation and was recovered from the human intestinal tract fifteen months ago. The other was KL very kindly supplied by Professor L. F. Rettger of Yale University. The most important distinction between the R and S variants from the clinical standpoint is that the R strain is of established therapeutic value while the therapeutic claims for the S strain remain unconvincing (Lynch, 1928).

PROCEDURE

Since viability is intimately associated with acidity we have used initial titratable acidities of 0.6 and 1.0 per cent for each of the strains used. The reason for this selection is that most commercial acidophilus milks have a titratable acidity of approximately 0.6 per cent, while our own R culture is usually administered at a titratable acidity of 1.0 per cent.

Milk cultures in 300 cc. amounts of the 3 strains were incubated at 35°C. until the desired total acidity was reached. For each strain two flasks at each acidity were held constant at 4°C. and two corresponding flasks for each acidity were held constant at 20°C.

At twenty-four-hour intervals 5 plates were poured from both the 1:100,000 and 1:1,000,000 dilutions of each flask. The KL strain was plated on Kulp and White's tomato-juice agar and incubated in an atmosphere of 10 per cent CO_2 while the other 2 strains were plated on Kulp and Rettger's casein-digest agar and incubated in air. Counts were made after six days' incubation at 35°C. with our new Petri dish holder (Kopeloff and Blackman, 1931). In all cases the final count recorded was the average of 5 plates.

RESULTS

The results of storing acidophilus milk of 1 per cent titratable acidity made with the rough strain are incorporated in table 1. In this and succeeding tables counts are expressed in millions but the 6 ciphers are always omitted. Experimental error has been calculated according to the formula:

$$\pm 0.6745 \sqrt{\frac{\Sigma D^2}{n(n-1)}}$$

It will be seen that in table 1 the results for each set of flasks have been tabulated separately since the daily platings on one set of flasks were always made by the same investigator while the daily platings on the duplicate flasks were always made by another investigator. After three days in the ice-box, both flasks A and B showed that over 90 per cent of the original organisms had died. This again confirms our previous findings. In the present report our average experimental error on 5 plates of a single dilution was ±4.20 per cent. The left half of plate 1 brings out clearly the comparison between the loss of viability of the rough acidophilus milk at ice-box and room temperatures.

In our tables there is no definite correlation between fluctuation in numbers of viable organisms and titratable acidity. This is undoubtedly due to the complexity of the determining factors

TABLE 1

Rough acidophilus milk—1 per cent titratable acidity

(Count in millions)

STORAGE	FLASKS A								FLASKS B							
	Ice-box				Room				Ice-box				Room			
	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count
days	*per cent*				*per cent*				*per cent*				*per cent*			
0	1.03	396	1.06		1.07	403	1.62		1.03	461	1.57		1.06	435	1.45	
1	1.08	370	1.78	93.4	1.06	438	1.26	108.7	1.03	355	10.63	77.0	1.06	419	3.96	96.3
2	1.04	8	2.16	1.9	1.02	306	11.05	75.9	1.05	62	3.46	13.4	1.08	230	13.38	52.9
3	1.05	18	3.53	4.5	1.06	104	14.41	25.8	1.08	27	11.05	5.9	1.16	347	2.91	80.0
4	1.03	23	0.68	5.8	1.01	135	2.53	33.5	1.10	32	8.02	6.8	1.09	215	3.06	49.4
6	1.07	10	14.00	2.5	1.15	116	3.67	28.8	1.10	9	17.04	1.9	1.12	263	3.79	60.5
9½	1.07	1	10.10	0.3	1.11	57	2.10	14.1	1.07	9	7.89	2.0	1.11	197	3.14	45.3

involved. There may even be some question as to the accuracy of titratable values when using 1-cc. samples of milk.

The smooth acidophilus milk (1 per cent acidity) also showed diminished viability in the ice-box as compared with room temperature. This is apparent from an examination of table 2. However, one is struck by the fact that after the first day's storage at either ice-box or room temperature, the numbers of viable organisms have actually increased over those initially present.

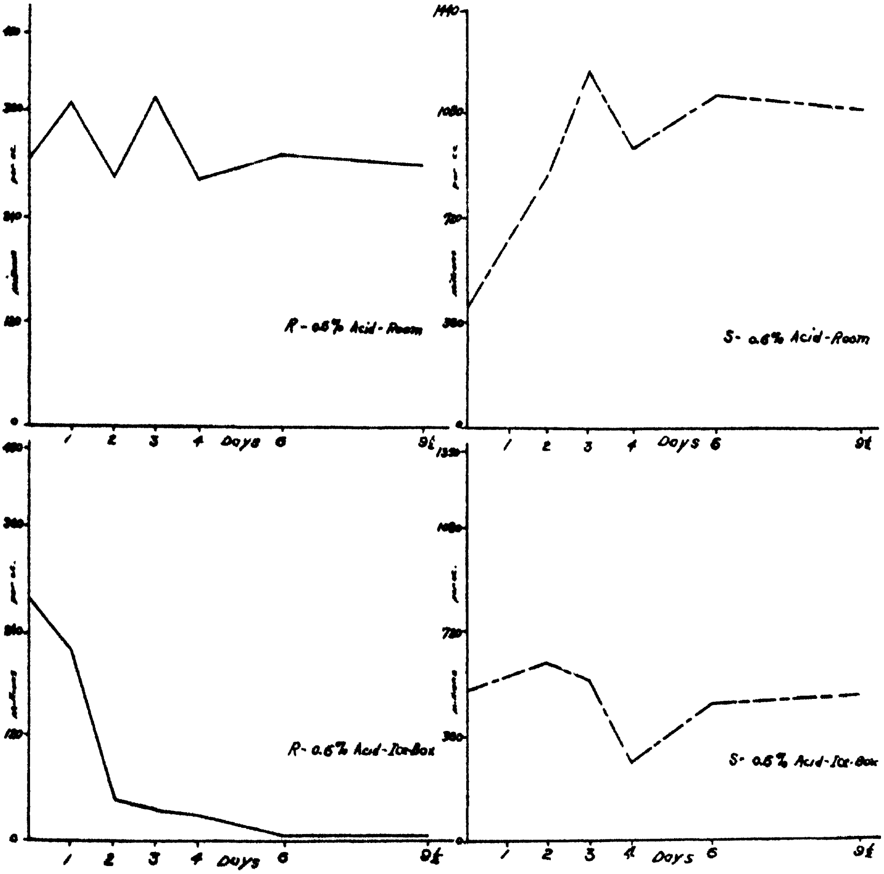

PLATE 1

Viability of Rough (R) and Smooth (S) Acidophilus 1 Per Cent Acidity at Ice-box and Room Temperature

Duplicate flasks indicated by solid and dotted lines

TABLE 2

Smooth acidophilus milk—1 per cent titratable acidity

(Count in millions)

STORAGE	FLASKS C								FLASKS D							
	Ice-box				Room				Ice-box				Room			
	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count
days	*per cent*				*per cent*				*per cent*				*per cent*			
0	0.98	1104	1.10		1.03	1114	1.51		1.06	1165	2.16		1.05	1361	1.34	
1	1.13	1208	5.01	109.4	1.25	1203	3.04	108.0	1.18	1317	1.60	113.0	1.33	1404	1.20	103.2
2	1.09	296	9.98	26.8	1.42	871	2.00	78.2	1.23	746	10.95	64.0	1.42	1007	3.08	74.0
3	1.22	479	7.47	43.4	1.55	1103	3.26	99.0	1.21	877	2.15	75.3	1.66	963	1.11	70.8
4	1.14	311	2.14	28.2	1.60	1125	0.60	101.0	1.22	844	2.93	72.4	1.65	1366	1.24	100.4
6	1.22	117	8.93	10.6	1.64	932	1.98	83.7	1.30	861	1.48	73.9	1.80	1124	0.93	82.6
9½	1.20	575	2.30	52.1	1.85	707	2.87	63.5	1.27	647	12.07	55.5	1.96	557	4.57	40.9

The subsequent decrease is not nearly so abrupt as with the rough acidophilus milk. The counts from the two flasks of smooth acidophilus were not in close agreement. The right half of plate 1 brings out these relationships graphically.

An interesting feature of these graphs is the marked secondary acceleration of growth in the smooth strain of acidophilus either at ice-box or room temperature and the secondary acceleration to be noted in the rough strain at room temperature.

The results obtained upon storing rough and smooth acidophilus milk of 0.6 per cent acidity in the ice-box and at room temperature are recorded in table 3.

From the left half of table 3 it will be seen that in the ice-box the rough acidophilus milk of 0.6 per cent acidity lost its viability very rapidly. At the end of three days' storage almost 90 per cent of the organisms had died. At room temperature, on the contrary, the viability was diminished to a very slight degree only. In fact the count increased after one and three days respectively. Again then, we may say that at either 0.6 or 1 per cent titratable acidity viability of the rough strain was seriously impaired in the ice-box but only slightly affected at room temperature.

In the right half of table 3 it will be seen that the smooth acidophilus milk of 0.6 per cent acidity behaved differently from the rough strain. Thus, even in the ice-box there was an increase in count after three days, while at room temperature there was a still more striking increase. The marked difference between the behavior of rough and smooth strains of 0.6 per cent acidity, shown graphically in plate 2, may be accounted for as follows. Inasmuch as the smooth strain used was a hardier organism which normally attained higher numbers at any given acidity, on continued storage at any but completely inhibitory temperatures the result was an attempt to establish a maximum population. Or to put it another way, the rough strain at 0.6 per cent acidity was nearer its maximum development than was the smooth strain. Consequently, continued storage resulted in a relatively greater increase in the smooth strain at room temperature and a relatively smaller diminution in the ice-box. Although duplicate

TABLE 3

Rough and smooth acidophilus milk—0.6 per cent titratable acidity

(Count in millions)

	ROUGH									SMOOTH							
	Ice-box				Room					Ice-box				Room			
Storage	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Storage	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count
days	*per cent*				*per cent*				*days*	*per cent*				*per cent*			
0	0.62	283	1.21		0.63	307	1.85		0	0.64	517	2.47		0.63	405	0.79	
1	0.70	219	5.08	77.4	0.85	370	1.54	120.5	1	0.72	478	5.15	92.5	0.87	545	5.53	134.6
2	0.78	48	18.71	17.0	0.77	285	6.08	92.8	2	0.83	624	5.34	120.7	1.10	883	2.26	218.0
3	0.74	35	2.92	12.4	0.83	375	1.65	122.1	3	0.85	570	2.00	110.3	1.16	1222	0.68	301.7
4	0.79	29	3.08	10.1	0.85	282	1.34	91.9	4	0.90	280	12.40	54.2	1.33	966	3.98	238.5
6	0.79	5	1.13	1.8	0.83	307	2.04	100.0	6	0.90	467	4.16	90.3	1.49	1149	1.66	283.7
9½	0.77	7	6.83	2.7	0.90	299	0.95	97.4	9½	0.90	511	3.08	98.8	1.77	1014	2.51	250.4

flasks were inoculated for this as for the previous experiments, in table 3 only single flask determinations are recorded owing to an unfortunate laboratory accident.

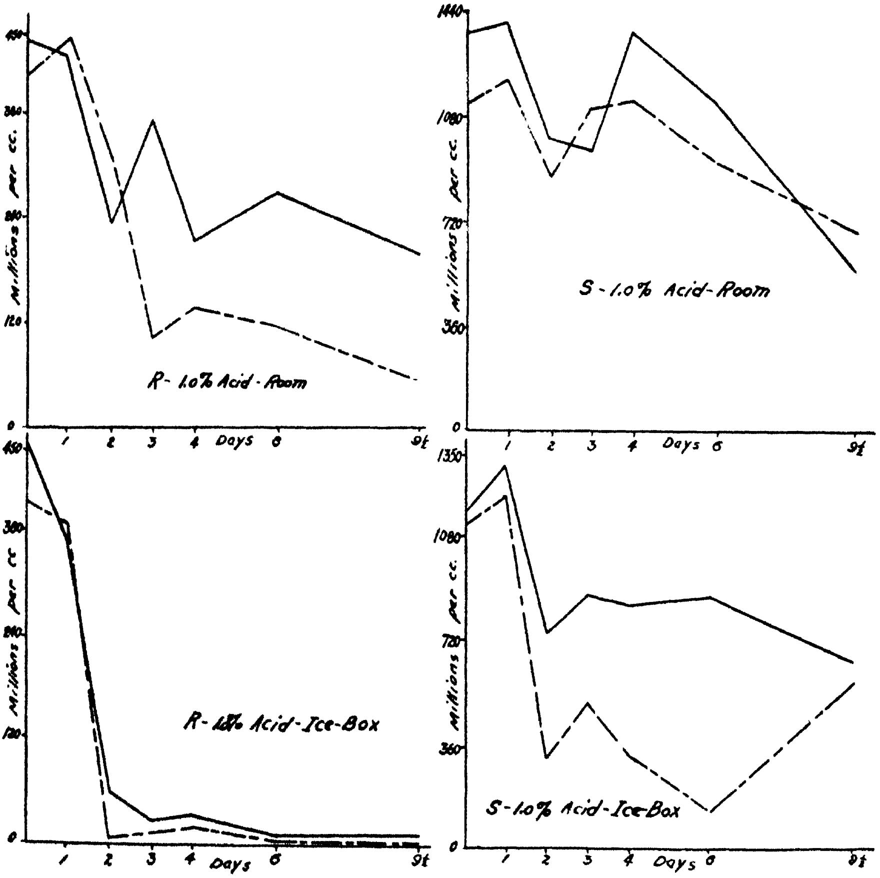

PLATE 2

Viability of Rough (R) and Smooth (S) Acidophilus 0.6 Per Cent Acidity at Ice-box and Room Temperature

In table 4 we have presented the data for the other rough strain, KL. It is to be regretted that we were unable at this time to employ more strains of both types since there must be considerable individual variation. Mixtures of rough and smooth strains would probably behave differently from either pure rough or smooth strains. This may well account for the discrepancies

TABLE 4

Rough acidophilus milk—KL strain—0.6 per cent titratable acidity

(Count in millions)

STORAGE	FLASKS A								FLASKS B							
	Ice-box				Room				Ice-box				Room			
	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count	Titratable acidity	Count per cubic centimeter	Per cent error	Per cent of original count
days	*per cent*				*per cent*				*per cent*				*per cent*			
0	0.62	229	3.15		0.63	207	5.42		0.67	203	1.27		0.67	223	1.61	
1	0.61				0.64	194	2.77	93.7	0.69	194	5.67	95.6	0.71	228	5.32	102.2
2	0.65	106	4.93	46.3	0.62	224	3.10	108.2	0.68	103	2.96	50.7	0.68	201	1.45	90.1
3	0.67	94	2.84	41.0	0.66	162	3.93	78.3	0.71	72	3.09	35.5	0.72	171	2.70	76.7
4	0.69	74	4.19	32.3	0.69	156	4.01	75.4	0.72	51	4.91	25.1	0.75	100	4.53	44.8
6	0.65	51	4.23	22.3	0.70	119	4.03	57.5	0.66	33	2.14	16.3	0.71	124	3.02	55.6
10	0.66	12	8.24	5.2	0.72	144	0.31	69.6	0.67	24	5.44	11.8	0.75	112	3.76	50.2

between the results obtained by different investigators since a preponderance of smooth organisms would probably not show as striking a diminution in viability as would a larger number of rough organisms.

Table 5 is a comparison of our present results with those previously published on acidophilus milk of approximately 1 per cent titratable acidity. While the data are not so clear cut as to make possible a dogmatic statement as to the influence of temperature upon viability, nevertheless it will be seen that with the rough acidophilus milk the count decreased much more rapidly at the lower than at the higher temperatures. Curiously, the viability at 20°C. diminished less rapidly than at 25°C. after the first two days. This may have been due to an increased production of inhibitory metabolites at the higher temperature. The smooth acidophilus culture did not lose its viability as rapidly as

TABLE 5

Acidophilus milk (1 per cent acidity) at different temperatures of storage

	ICE-BOX				ROOM			
	4°C.		9°C.		20°C.		25° to 30°C.	
Strain	R	S	R	S	R	S	R	S
Initial acidity, per cent	1.03	1.02	1.13		1.06	1.04	1.12	
Initial count	428	1134	197		419	1238	177	
Storage	Per cent of original count							
days								
1	85.2	111.2	54.3		102.5	105.6	104.0	
2	7.7	45.4	28.8		64.4	76.1	79.7	
3	5.2	59.4	12.9		52.9	84.9	24.9	
4	6.3	50.3	6.0		41.5	100.7	7.3	
5			0.3				17.0	
6	2.2	42.3	3.0		44.7	83.2	6.8	
7							5.1	
8							10.7	
9							11.3	
9½	1.2	53.8			29.7	52.2		
10							14.1	
11							1.4	

did the rough acidophilus culture at either ice-box or room temperature. Nevertheless, after the first day's storage the viability of the smooth acidophilus culture was greatly diminished in the ice-box as compared with room temperature. After nine days' storage they both tended to reach the same count. This finding agrees with results in Kulp's table 1 (Kulp, 1931). A final decision as to the optimum temperature at which to store acidophilus milk would have to depend upon the strain of *L. acidophilus* employed, the count attained, the initial acidity and the number of viable organisms desired for consumption.

SUMMARY

Subject to the limitations of the material at hand, it has been shown that:

1. The viability of rough and smooth strains of acidophilus milk of the same titratable acidity differed at 4° as well as at 20°C.
2. The rough strain of acidophilus milk, which is the only one of proved therapeutic value, lost its viability rapidly at 4° or 9°C. as compared with storage at 20° or 25° to 30°C. With an initial titratable acidity of 0.6 or 1.0 per cent the original count diminished approximately 90 per cent in three days in the ice-box and between 20 and 75 per cent at room temperature.
3. The smooth strain of acidophilus milk used lost its viability less rapidly than the rough strain but storage in the ice-box was more detrimental than at room temperature.

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LACTOBACILLUS BIFIDUS[1]

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The Lactobacillus genus constitutes one of the most widely distributed groups of known microörganisms. The group as a whole is primarily fermentative in character, and displays a marked degree of tolerance for acids, hence the term "aciduric," which is now generally applied to it. Members of this genus vary widely in their physiological activities and in their habitat. Some are characteristically milk and dairy products types; others are intimately associated with raw cereals and vegetables; others are of oral and dental origin; and still others are primarily intestinal in type. The intestinal types (*L. bifidus* and *L. acidophilus*) constitute the material for the present study.

An intensive review of the literature on aciduric bacteria of the intestine reveals almost hopeless confusion concerning the nature and biological position of *L. bifidus* of Tissier and, in spite of the large number of papers that have been published, its entity is still far from being established. For a more extensive review of the literature than space here permits the reader is referred to the senior author's doctorate thesis deposited in the Yale University Library.

Tissier (1899–1900) first isolated and described this organism and, because of its tendency to branch, called it *B. bifidus*. He classified it as a non-motile, non-spore-forming anaerobic pleomorphic rod which is Gram-positive in young cultures. In 1905 Tissier reported finding *L. bifidus* as the predominating organism in breast-fed, and *L. acidophilus* in bottle-fed, children.

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[1] This paper covers in part the work submitted to the Graduate School of Yale University by the senior author as part requirement for the degree of Doctor of Philosophy.

Moro (1900) reported the isolation of a Gram-positive rod from the stools of breast-fed infants which had many points of resemblance with *L. bifidus*. Because of its unusual acid tolerance, he called it *B. acidophilus*. Moro was of the opinion that his *B. acidophilus* was the predominant organism in the feces of breast-fed infants, and for a long time held to this view. Rodella (1908) could find no differences between the Boas-Oppler bacillus, *B. bifidus*, and *B. acidophilus*, in so far as their morphological and cultural characteristics were concerned. He found branching forms of *B. acidophilus*.

Many later investigators claimed to have isolated *L. bifidus*, but very few have given any detailed description of the organism. It has been described as an anaerobe, an aerobe, as branching, and as not branching; it has been placed by some in the *Lactobacillus* genus; by others among the bacteroides and again by others in the coli-typhi group.

Among the more recent contributions dealing with *L. bifidus* as a definite entity is that of Cruickshank (1925). This author reported finding *L. bifidus* present regularly in the stools of breast-fed infants, at times composing almost 99 per cent of the flora. He stated that *L. bifidus* is a strict anaerobe in primary culture, but that thereafter it grows readily in the presence of free oxygen. He described its varied morphology, but did not stress branching, thinking that this was unusual. He believed that the organism closely resembles *B. acidophilus*, but that it differs from the latter in morphology and certain cultural characteristics. According to him, *L. bifidus* is not a well-defined group serologically.

ISOLATION OF LACTOBACILLUS BIFIDUS

The following methods were employed: (1) that of Tissier (1899–1900) which involves the use of glucose-infusion agar in Veillon tubes; (2) the procedure of Torrey (1917) in which acid liver-infusion glucose agar to which 10 per cent of rabbit's blood is added is employed; (3) the Stransky and Maslowski (1927) modification of the Adam method (1921–22–23), which makes use of milk whey containing marble chips, followed, after twenty-four hours' incubation, by inoculation into deep glucose agar tubes, and (4) the tomato-broth isolation method devised in this laboratory and based largely on the tomato-broth medium of Kulp (1927).

Since the tomato medium, both with and without agar, proved to be particularly useful and satisfactory in this investigation, the method of its preparation is given here briefly.

Preparation of tomato-broth medium. The contents of a large can of tomatoes are filtered through coarse filter paper. The clear filtrate is diluted with distilled water, 800 cc. of water being added to 200 cc. of the filtered tomato juice. To this are added 1 per cent peptonized milk (Difco), 0.5 per cent yeast extract (Difco), and 0.5 per cent peptone (Difco). The medium is adjusted to pH 8.0, tubed and autoclaved. For use as agar medium, 2 per cent Difco granular agar is dissolved in the liquid medium. The tubes are sterilized by autoclaving for fifteen minutes at 15 to 18 pounds extra pressure. The final pH of the broth and agar media is 6.8, the autoclaving having a definite pH depressant effect.

The tomato broth and agar offer a particular advantage in the isolation of intestinal lactobacilli in that they contain favorable food, especially a liberal amount of available carbohydrate which furnishes sufficient acid during the cultural period to depress the pH to 3.6 to 4.0, a level at which the lactobacilli are not destroyed, while other intestinal bacteria are held in check or actually killed.

Isolation procedure. A liberal amount of nursling's stool is placed in the tomato broth and the inoculated tubes held under anaerobic conditions at 37°C. for one week. Anaerobiosis is obtained by the use of anaerobic jars and a vacuum pump. At the end of the incubation period a loopful from each tube is streaked over the surface of tomato agar in Petri plates, which are then incubated anaerobically at 37°C. for seventy-two hours. Typical colonies are selected and grown in tomato broth. Alternate restreaking of agar and growth in the liquid medium are continued until pure cultures are obtained. This method, although relatively slow, has proven to be thoroughly dependable for the isolation of *L. bifidus* from infants' feces. The Veillon tube method, while much shorter, does not lend itself well to isolation.

Most of the strains used in this investigation were obtained by the tomato-broth isolation method. Anaerobiosis was found to be necessary during isolation, and greatly facilitated growth during the next two or three transfers.

Sources of strains of L. bifidus. The fecal specimens from which all human strains were isolated were obtained from the Maternity Wards of the Philadelphia General Hospital. The human strains were numbered 1 to 31, in the order of isolation.

For comparison with the human strains, several strains of *L. bifidus* were obtained from white rats, according to the procedure of Rettger and Cheplin (1921). This method consisted in feeding the rats a diet high in lactose and making isolations by the Veillon tube and acid broth methods. The observations of Hull and Rettger (1914) and of Rettger and Cheplin that *L. bifidus* will develop in the intestine of rats receiving a high lactose diet, were confirmed; 13 strains were isolated from rat feces; these were numbered 1R to 13R, in the order of isolation. Attempts to isolate *L. bifidus* from the feces of normal monkeys, chimpanzees, baboons, horses, dogs, cats and cows were fruitless. Furthermore, no cultures of *L. bifidus* could be secured from other laboratories. Several strains of *L. acidophilus* were employed in this investigation. These were Scavano, R–1–1, R–1–5, TA, LWS and McGrath, all stock strains of this laboratory.

The stock strains of *L. bifidus* and *L. acidophilus* were kept in litmus milk, transfers being made every two weeks. *L. bifidus* grew well in this medium, the majority of strains producing curds within two to three days. In transferring the stock cultures, 1 cc. amounts were always used.

OXYGEN REQUIREMENTS

The literature appears to be about equally divided regarding the oxygen requirements of *L. bifidus*. Tissier (1899), Cahn (1901), Jacobson (1908), Distaso (1911), Logan (1913), Adam (1922), Stransky and Maslowski (1927) and others believed this organism to be a strict anaerobe. On the other hand, Passini (1903), Mereshkowsky (1905 and 1906), Noguchi (1910), Basten (1914), Kuthe (1915), Torrey (1917) Howe and Hatch (1917), Brown and Bosworth (1922), Webster (1923), Cruickshank (1925), Gerstly, Howell and Nagel (1932) and others concluded that *L. bifidus* is either an aerobe or a facultative anaerobe.

Like *L. acidophilus*, *L. bifidus* requires an unusually favorable medium for maximum growth. The lack of such a medium has, without doubt, been the reason why many investigators have concluded that *L. bifidus* is an anaerobe. It grows aerobically on a rich and suitable medium, except in primary culture, when it requires anaerobic conditions. It is expedient to continue the anaerobiosis for several transfers after isolation. Triplicate cultures placed under strictly anaerobic, aerobic and partially anaerobic (air and 10 to 20 per cent CO_2) conditions showed about equal growths, the partially anaerobic conditions proving slightly more favorable than the others. We may safely conclude that *L. bifidus* is a facultative anaerobe. In this respect it does not differ from *L. acidophilus*. Most of the present experimental work was carried out under partially anaerobic conditions.

MORPHOLOGY

According to Tissier and others, *L. bifidus* is decidedly pleomorphic, true branching, club and racquet forms being not at all uncommon. These claims, particularly those regarding true branching, have been disputed by various investigators; the morphology of *L. bifidus* received special attention, therefore, in this investigation.

Stained films made directly from the feces of breast-fed infants (fig. 1) reveal, as a rule, an almost pure picture (90 to 95 per cent) of slender, straight and comma-shaped, Gram-positive rods which vary from 2 μ to 8 μ in length (average 3μ to 5 μ), and from 0.5 μ to 0.7 μ in width. The rods possess rounded ends, with an occasional bulbous thickening at one extremity. The orientation of the individual cells is often such as to suggest branching in the form of a Y or T. Real branching may be seen at times; this is usually of the Y type. Chain formation is absent, or at best very rare. Some of the cells stain unevenly and thus assume a granular or beaded appearance; others may be completely Gram-negative.

Films prepared from pure young cultures in broth, milk and agar reveal, on the whole, a morphology somewhat similar to that observed directly in nursling's stools. The rods are more variable as to length, some approaching coccoid forms and others attaining 8 μ to 10 μ in length. The staining is quite uniform in young cultures, granular or beaded forms being not as common as in fecal slides. While the cells are distinctly Gram-positive, it is relatively easy to decolorize them completely with alcohol. There is some tendency to chain formation, but not so marked as that displayed by *L. acidophilus.* No true branching has been observed by us in pure broth, agar and milk cultures of *L. bifidus.* Most of the rods are curved, comma and S forms being quite common. Older cultures show more variable cells, which tend toward the filamentous type.

The microscopic picture presented by young and old cultures of *L. acidophilus* does not differ markedly from that of *L. bifidus.* Both show a variable morphology. There is a greater tendency toward chain formation by *L. acidophilus* in the young than in the older cultures; branching is extremely rare. This organism resembles *L. bifidus* further in that older cultures lose their Gram-positive character and tend to become filamentous. Furthermore, granule formation is quite common. It would be very difficult to differentiate *L. bifidus* from *L. acidophilus* on the basis of morphology.

Repeated failures to demonstrate true branching of *L. bifidus* led the writers to revert to the method employed by Tissier for the isolation and study of this organism. Accordingly, nurslings' feces were inoculated into Veillon tubes containing glucose infusion agar, and the tubes incubated at 37°C. for seventy-two hours. Stained slides were prepared from the small, white oval and lens-shaped colonies (fig. 2). The microscopic picture observed in such films resembled that so clearly described by Tissier, except that the rods possessed rounded, instead of pointed, ends. Various kinds of branching forms were of common occurrence, particularly the Y and T. The Y forms were the most numerous, and their ends were often distinctly thickened or club-shaped. Some cells exhibited branching and re-branching. While the branches were usually short, some attained considerable length.

Tissier's claims that *L. bifidus* reveals a decidedly varied morphology and true branching were further confirmed on examination of stained films prepared from primary colonies grown on Torrey's medium (fig. 3). The morphology of the cells in these colonies was even more extreme than that observed in the Veillon tubes, branching and re-branching being quite common. Subcultures prepared on the routine media from the Veillon tubes and the original platings with Torrey's medium showed little pleomorphism, and no branching.

It had been assumed by us that *L. bifidus* could be distinguished from *L acidophilus* on the basis of extreme pleomorphism and branching. However, when human feces containing *L. acidophilus* were inoculated into Veillon tubes containing glucose infusion agar, and the tubes incubated for seventy-two hours, branching forms were readily obtained from the acidophilus colonies (fig. 4). Many types of cells were seen, including small and large Y and T forms, as well as clubbed, nobbed, vesicular and other forms. In fact the microscopic picture presented by *L. bifidus* was duplicated here. These observations were repeated many times with different samples of feces.

CULTURAL CHARACTERISTICS

The colonies of *L. bifidus* have been described as being oval or lenticular, and smooth. Most investigators have confined their studies on colonial form to growth in deep glucose infusion agar. It has been observed, however, that when a medium is used which contains a readily utilizable carbohydrate, and in which the organism grows abundantly, *L. bifidus* produces more or less rough colonies which may resemble those of *L. acidophilus*.

In the present investigation *L. bifidus* was observed to form small, white, round or oval colonies in deep glucose beef-infusion agar in Veillon tubes, which could be seen at times only by means of a magnifying lens. The colonies are fairly smooth, and almost entirely solid; they usually possess a delicate plumose or filamentous border. Surface (streak) colonies on glucose beef-infusion agar are larger, quite granular, and have an irregular (not filamentous) edge. On tomato peptonized milk agar in poured plates the subsurface colonies (fig. 5) bear considerable resemblance to the xy type of *L. acidophilus*, being comprised of a solid center and filamentous border. The X or highly filamentous type of colony which is so common with *L. acidophilus* (fig. 6) has seldom been observed by us in *L. bifidus*. Many different factors influence the size of the colonies, such as crowding, length of time during which the strain has been artificially cultured, the medium used, temperature of incubation, and inherent peculiarities of the different strains. The fully developed colonies measure from 1 to 3 mm. in diameter.

Typical mature (seventy-two hours) surface colonies of *L. bifidus* on agar streak plates differ more or less, macroscopically, from those of *L. acidophilus*. The former appear quite solid, dark and granular, with a slightly irregular edge; they are disc-wheel-like and average from 2 to 3 mm. in diameter. *L. acidophilus* on the other hand, produces flat, thin, spreading colonies having a distinctly irregular or wavy edge, and are from 2 to 4 mm. in diameter. Under the microscope the colonies of the two types appear quite granular and possess a more or less plumose or filamentous border, the rough characteristic being much more pronounced in the acidophilus than in the bifidus colonies. (See figs. 7 and 8.)

L. bifidus produces a distinct growth in tomato broth in twenty-four hours, with general turbidity and a slight sediment formation. At the end of six days the broth is practically clear, the growth having settled out. The optimum temperature is 37°C. Very little or no growth occurs at 20° to 22°C. The 37° culture resembles that of *L. acidophilus* grown at the same temperature. No visible growth is produced by either organism in *sugar-free* meat extract, beef infusion or yeast-extract broth or agar during incubation at 37°C. for five days. In yeast-extract broth and agar containing galactose both organisms grow well.

Litmus milk was curdled at 37°C. by nearly all of the strains of *L. bifidus*, and by all of the strains of *L. acidophilus*. The length of time required for curdling varied from twenty-four hours to several days. *L. acidophilus* regularly produced a solid curd and a pleasant sour odor. Only about half of the *L. bifidus* strains developed a solid curd, while the remainder formed a more or less lumpy curd. Torrey claimed to be able to differentiate *L. bifidus* from *L. acidophilus* by the growth produced on glucose blood liver-infusion agar. In our study the two organisms showed about the same type of colony, except that *L. bifidus* usually produced a greenish zone around the colony, while *L. acidophilus* did not. However, this difference may have been due to the more rapid development of *L. bifidus* on this medium.

FERMENTATION STUDIES

In these studies 31 strains of *L. bifidus* and 5 of *L. acidophilus* were used against twenty-one sugars and alcohols. The sugars were sterilized as 10 per cent water solutions, and added aseptically to yeast extract broth. Galactose, inulin, xylose, arabinose, levulose and rhamnose underwent a slight breakdown on steam sterilization; hence, solutions of these sugars were sterilized by filtration. The inoculums were prepared by centrifuging the growth obtained in tomato broth, washing with sterile physiological salt solution, recentrifuging and rewashing until the washings were neutral. The cells were then suspended in saline solution, each suspension being made up to a turbidity of 2.0 on the McFarland nephelometer scale, and 0.5 cc. of this suspension added to each medium. Brom-thymol-blue was employed as indicator. Uninoculated controls were run for each set of sugars, as well as an inoculated tomato-broth tube, to check the purity of each strain. Readings were made at twenty-four, forty-eight and seventy-two hours, and after one week. All of these tests were carried out twice, with a three months' interval between tests.

All of the strains of *L. bifidus* fermented galactose, inulin and levulose; nearly all attacked sucrose; a large majority formed acid in starch, dextrin, maltose, glucose, raffinose and trehalose. Some strains broke down lactose and salicin readily; an occasional strain attacked mannitol, glycerol and rhamnose. None acted upon amygdalin, xylose, dulcitol, arabinose, inositol and melizitose. When the results of the fermentation tests with *L. acidophilus* are compared with those of *L. bifidus*, only one difference is observed; a greater proportion of the strains of *L. acidophilus* than of *L. bifidus* visibly attacked lactose. This may have been due, however, to the fact that the acidophilus strains have long been kept in milk and have therefore developed a pronounced lactose-splitting power. Some strains of *L. bifidus* differed markedly from some strains of *L. acidophilus*, in their carbohydrolytic properties, but at the same time different strains of *L. bifidus* differed materially from each other. With such differences between strains of the same species, one could hardly hope to differentiate these two organisms by the fermentation test. No gas formation occurred. The sugar-containing broths which were not fermented showed no growth; hence, growth in itself should be a good indication of the utilization of a carbohydrate by these two closely related organisms.

AGGLUTINATION TESTS

The immunological reactions of *L. bifidus* have been studied very little, and most of the investigators who made such studies used only a few strains, and generally only one antiserum. For the most part, the results seem to point to the fact that *L. bifidus* strains vary immunologically among themselves, and show a close general relationship to *L. acidophilus*. In the present investigation 12 antisera were prepared (9 of *L. bifidus* and 3 of *L. acidophilus*). These antisera were obtained in the usual way, rabbits receiving 3 injections weekly of large amounts of washed cells (dead and alive) in the marginal ear vein until a total of 16 injections had been given. Ten days after the last injection the rabbits were bled from the heart, and the serum separated and preserved with phenol. Antigens were prepared in distilled water, after some experimentation with physiological salt and various buffer solutions. The distilled water was found to be the most satisfactory for holding the lactobacilli in suspension. It may be objected to on the grounds that it is relatively free

TABLE 1

Showing agglutination results

STRAINS	SERA											
	1	2	6	7	9	17	27	31	5R	Scav	LWS	TA
						L. bifidus						
1	5+	3+	–	4+	2+	–	3+	3+	2+	2+	2+	5+
2	1+	5+	AC	AC	3+	AC	2+	3+	3+	2+	2+	4+
3	2+	4+	2+	3+	–	1+	2+	2+	1+	1+	2+	3+
4	2+	4+	3+	2+	3+	–	2+	3+	4+	3+	2+	4+
5	–	3+	5+	3+	–	4+	2+	4+	4+	4+	4+	4+
6	1+	AC	5+	1+	2+	AC	AC	AC	AC	AC	AC	AC
7	4+	–	–	5+	2+	–	–	2+	–	–	3+	4+
8	2+	4+	3+	3+	2+	2+	4+	2+	2+	2+	2+	1+
9	4+	–	2+	2+	5+	4+	2+	–	–	–	1+	1+
10	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
11	5+	5+	3+	–	2+	2+	2+	–	2+	–	2+	5+
12	5+	5+	–	1+	–	–	–	–	–	–	–	5+
13	5+	5+	–	3+	2+	–	–	1+	–	–	1+	4+
14	5+	–	–	–	–	–	–	2+	–	3+	3+	–
15	5+	5+	2+	3+	3+	1+	–	1+	–	–	–	–
16	5+	5+	2+	2+	–	4+	–	2+	2+	2+	–	4+
17	2+	–	3+	–	–	5+	–	2+	1+	–	–	–
18	5+	4+	5+	2+	–	2+	–	2+	1+	2+	–	–
19	3+	2+	–	2+	2+	1+	1+	–	–	–	–	–
20	1+	–	–	2+	–	–	2+	2+	4+	1+	–	–
21	1+	–	–	2+	–	3+	4+	2+	–	2+	2+	–
22	2+	–	2+	1+	–	–	–	3+	1+	2+	2+	–
23	2+	–	3+	4+	–	–	–	2+	1+	2+	3+	1+
24	5+	–	3+	2+	–	–	–	2+	2+	1+	2+	–
25	4+	5+	2+	2+	–	3+	–	–	–	–	–	4+
26	–	–	–	3+	1+	3+	–	–	–	–	2+	–
27	–	–	–	5+	–	–	5+	2+	2+	–	–	2+
28	AC	AC	AC	2+	AC	AC	AC	2+	AC	AC	–	AC
29	AC	AC	AC	5+	AC	4+	AC	4+	3+	2+	2+	1+
30	2+	–	AC	2+	AC	AC	4+	2+	–	5+	3+	2+
31	–	–	–	1+	1+	1+	3+	5+	2+	2+	2+	3+
1R	–	–	–	1+	3+	1+	1+	2+	4+	2+	2+	3+
2R	–	–	1+	2+	1+	1+	1+	4+	4+	2+	3+	3+
4R	–	–	1+	–	1+	1+	1+	4+	4+	2+	3+	1+
5R	–	–	5+	–	2+	–	–	5+	5+	2+	3+	3+
						L. acidophilus						
SCav	–	–	–	–	–	1+	–	–	1+	5+	–	–
LWS	5+	5+	–	–	–	1+	–	–	2+	2+	5+	5+
TA	5+	5+	–	–	–	1+	–	–	1+	2+	4+	5+

Key: 1+, agglutination 1:20; 2+, agglutination 1:40; 3+, agglutination 1:80; 4+, agglutination 1:160; 5+, agglutination 1:320 or higher; AC, antigen clumpy (no reading possible); –, negative.

from electrolytes, but the water used here was only once-distilled and was far from being pure. Antisera for the following organisms were used: *L. bifidus*, No. 1, 1:1280; No. 3, 1:1280; No. 6, 1:640; No. 7, 1:640; No. 9, 1:2560; No. 17, 1:640; No. 27, 1:640; No. 31, 1:640; and strain 5R, 1:640; *L. acidophilus*, *Scavano*, 1:320; strain LWS, 1:320; and strain TA, 1:1280.

These antisera were set up against 36 strains of *L. bifidus* and 3 strains of *L. acidophilus*, starting with a dilution of 1:20 and doubling for each of 10 successive dilutions. Readings were made after twenty-four hours at 37°C. The results of the serological tests are given in table 1.

As has been emphasized by other investigators, *L. bifidus* does not comprise a single antigenic species. There is a very close general relationship between *L. bifidus* and *L. acidophilus*, antigenically. The results of these tests show, however, that there is so much variation serologically between different strains of each of these two organisms that agglutination tests cannot be used to distinction them.

INFLUENCE OF H-IONS ON GROWTH

Adam (1923) stated that *L. bifidus* will grow in a pH range of 4.2 to 8.6, while the growth limits of *L. acidophilus* are pH 4.1 and 7.2. In the present study of H-ion influence a series of tubes containing glucose (1 per cent), yeast extract (0.5 per cent) and peptone (1 per cent) and ranging from pH 3.6 to 9.0, were treated with 0.5 cc. of a suspension of washed bacterial cells. They were incubated at 37°C., and growth recorded after twenty-four, forty-eight and seventy-two hours, and one week.

The average limiting pH for *L. bifidus* was found to be 4.4, and for *L. acidophilus* pH 4.6. A few strains of *L. bifidus* grew as low as pH 3.8, and some of the *L. acidophilus* strains at pH 4.0. *L. bifidus* did not grow beyond pH 7.2, as a rule, although a few strains slightly exceeded this figure. *L. acidophilus* revealed an average upper pH limit of 6.8. Repeated observations showed the average optimum pH for growth of *L. bifidus* to be between 5.4 and 6.4, and for *L. acidophilus* between 5.8 and 6.6.

BACTERIOSTATIC ACTION OF DYES

Cruickshank claimed that *L. bifidus* tolerates a concentration of 1:100,000 of crystal violet, while a solution of 1:1,000,000 of this same dye definitely inhibits *Staphylococcus albus.* Since the former organism is Gram-positive, one would assume that it is quite sensitive to crystal violet and closely related dyes. We attempted to test Cruickshank's conclusions, using glucose yeast-extract agar as the basic medium. Four Petri dishes were poured, containing different concentrations of crystal violet, namely 1:50,000, 1:100,000, 1:500,000 and 1:1,000,000 in one series, and basic fuchsin in another. The plates were streaked with liquid cultures of *L. bifidus, L. acidophilus, Staph. citreus* and *Bact. cloacae.* Control plates containing no dye were used in each case. There was complete or almost complete inhibition of growth of *L. bifidus* and *L. acidophilus* in all dilutions up to 1:1,000,000. *Staph. citreus* was inhibited definitely, showing a very slight growth in the 1:1,000,000 plates only, while *Bact. cloacae* was not inhibited in any of the concentrations of crystal violet employed. Basic fuchsin partially inhibited *L. bifidus, L. acidophilus* and *Staph. citreus* in dilutions of 1:50,000 and 1:100,000, and exerted little or no effect in the higher dilutions; *Bact. cloacae* was not inhibited at all. There was very good growth in all of the control plates. *L. bifidus* and *L. acidophilus* reacted as one would expect them to do towards crystal violet and related basic dyes, that is, like Gram-positive bacteria generally. Furthermore, the dye resistance tests revealed no difference in the tolerance of these two aciduric organisms for these dyes.

INDOL AND PHENOL TOLERANCE

Kulp (1929) showed that *L. acidophilus* offers a much greater resistance to indol and phenol than *L. bulgaricus.* In comparing the resistance of *L. bifidus* and *L. acidophilus* to these two agents, the methods of conducting the tests and the concentrations of indol and phenol which we used were the same as those of Kulp, except that yeast-extract nutrient agar containing 1 per cent glucose was employed by us in place of casein digest agar. Adequate control plates were included in the set up. The results obtained with *L. acidophilus* and *L. bifidus* were almost identical with those of Kulp for *L. acidophilus*. No appreciable difference could be observed between the resistance of the two organisms to the indol and phenol. Growth was inhibited by indol concentrations of 1:1100 and above, and complete tolerance was shown in concentrations of 1:2000 and less. Phenol in concentrations of 1:250 and above inhibited growth; normal growth occurred in concentrations of 1:400 and less. These results bear out the assumption that intestinal organisms are relatively resistant to the action of indol.

SEARCH FOR BACTERIOPHAGE

It was interesting to note that some strains of *L. bifidus* underwent complete lysis in broth during three days' incubation. This suggested bacteriophage action and led to a search for bacteriophage in the lactobacilli. Attempts to develop a phage from broth culture filtrates and from filtrates from suspensions of the feces of a breast-fed infant, for *L. bifidus*, *L. acidophilus*, *L. odontolyticus*, *E. coli*, *Proteus vulgaris* and *Staph. aureus*, resulted in failure. There was demonstrated, however, a definite inhibitory agent in broth filtrates of *L. bifidus*, which was sufficiently active to inhibit appreciably the growth of *L. bifidus*, and *L. acidophilus* when only one drop of filtrate was employed in 3 cc. of medium. This action could not have been due to acid. Many attempts to demonstrate the presence of filterable forms in the filtrates proved unsuccessful.

LYSOZYME STUDY

The lytic principle in the broth cultures could not be explained on the basis of bacteriophage action. It was observed that the lysis of cultures disappeared after the strains had gone through several transplants. This suggested the occurrence of a lysozyme, a substance first reported by Fleming and Allison (1922), and supposed to be present in all human tissues and in human secretions, except urine, sweat and cerebrospinal fluid. This substance or agent has the power to lyse certain bacteria and, according to Rosenthal and Lieberman (1931), is present in large quantities in the feces of breast-fed infants, having its origin in the human milk. These authors concluded that *L. bifidus* predominates in the feces of breast-fed infants because it is resistant to lysozyme action, while the other fecal forms are lysed by it.

Since egg-white is supposed to contain large amounts of lysozyme, fresh egg-white was added to liquid cultures of *L. bifidus* and *L. acidophilus.* No lysis occurred. Negative results were also obtained when egg-white was dropped on colonies in streaked plates. Egg-white, however, did inhibit the growth of these two organisms, which is in accord with the observations of Rettger and Sperry (1912). This appeared to support the claims of Rosenthal and Lieberman; but, when egg-white was tried against newly isolated strains of *E. coli* and the enterococcus no lysis occurred, either in broth, plates or washed suspensions. According to these observations, lysozyme plays no important, if indeed any, rôle in the predominance of *L. bifidus* in the feces of breast-fed infants. It also appears that lysozyme is not active against any of the ordinary intestinal organisms, and that it shows its lytic power only against special susceptible strains such as the "*Micrococcus lysodeikticus*" of Fleming.

LACTIC ACID PRODUCTION BY L. BIFIDUS

The investigation was almost completed up to this point when the paper of Curran, Rogers and Whittier (1933) on "The Distinguishing Characteristics of Lactobacillus Acidophilus" appeared in this JOURNAL. The properties ascribed by these authors to *L. acidophilus* agreed, on the whole, with those of *L. bifidus* as observed in the present study. Since considerable emphasis was placed by Rogers and his associates on the types and quantity of lactic acid formation by lactobacilli as a basis of classification, we undertook to make a roughly quantitative study of the acids produced by *L. bifidus.*

The methods described by Pederson, Peterson and Fred (1926) and by Pederson and Breed (1928) were employed. The following is a brief summary of the results obtained. The strains of *L. bifidus* isolated by us produced a total acidity varying (with the different strains) from 0.05 to 0.4 per cent, in terms of lactic acid. The volatile acid fraction varied between 18 and 25 per cent of the total; it consisted largely of acetic acid, with small amounts of formic and butyric. Lactic acid constituted from 75 to 80 per cent of the total acid. The lactic acid proved to be of the inactive type.

These results agree in the main with those of Curran, Rogers and Whittier for *L. acidophilus.* The total acid production by *L. bifidus* was, on the whole, less than that of *L. acidophilus,* and the volatile acid fraction of *L. bifidus* was slightly higher than that of *L. acidophilus.* However, the differences cannot be regarded as significant, especially since some strains of both types yielded essentially the same results.

DISCUSSION AND CONCLUSIONS

L. bifidus may constitute from 90 to 95 per cent of the intestinal flora of breast-fed infants. It appears in the feces in from three to four days after birth and remains the predominating organism as long as breast milk is the sole diet. The results of this investigation indicate a very close relationship between this organism and *L. acidophilus* of Moro.

L. bifidus is isolated most readily by the acid broth preliminary enrichment method but, in order to observe its quantitative relationship to other intestinal bacteria, direct plating of the feces in special agar media, under anaerobic conditions is necessary. After primary isolation it grows under ordinary aerobic conditions, and in pure culture. *L. bifidus* should be classed, therefore, as a facultative anaerobe.

The morphological and cultural characteristics of the two organisms are essentially the same, the few differences observed being only of a quantitative nature.

While *L. bifidus* seemed to possess a slightly wider carbohydrate-decomposing range than *L. acidophilus,* there was no significant difference between the two types, in so far as fermentation properties are concerned.

There are apparently no greater serological differences between

L. bifidus and *L. acidophilus* than exist between members of one and the same species.

Both organisms reacted practically alike to acid, alkali, phenol, indol, crystal violet, basic fuchsin and lysozyme.

Qualitative and quantitative studies of the acids formed by *L. bifidus* agree in general with those reported by Rogers and his associates for *L. acidophilus*. The bulk of the acids produced by both types of organisms is inactive lactic acid.

While there are, on the whole, slight differences between *L. bifidus* and *L. acidophilus*, these can be accounted for by normal variation within the species or type. The authors are of the opinion that *L. bifidus* should be regarded as a variant of the species in which *L. acidophilus* is the central type.

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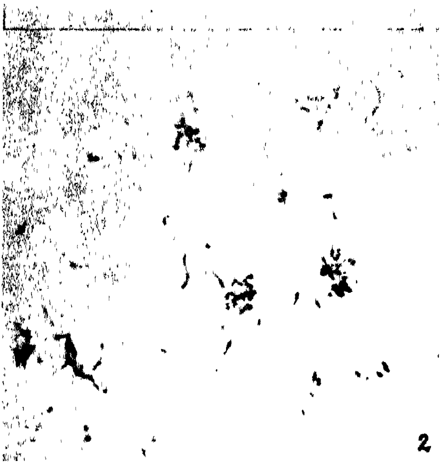
PLATE 1

Fig. 1. *L. bifidus.* Gram-stained preparation made directly from nursling's stool. × 1000.

Fig. 2. *L. bifidus.* Gram-stained preparation made from colonies in Veillon tube. Branching and club-shaped forms. × 1000.

Fig. 3 *L. bifidus.* Gram-stained preparation made from colonies on Torrey's medium showing extreme branching and re-branching. × 1000.

Fig. 4. *L. acidophilus.* Gram-stained preparation made from colonies in Veillon tube showing branching and re-branching. × 1000.



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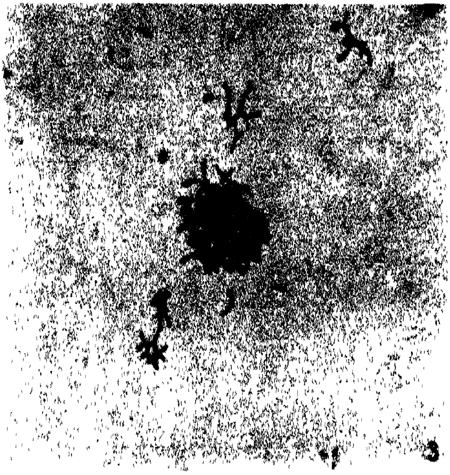

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(James E. Weiss and Leo F. Rettger: Lactobacillus bifidus)

PLATE 2

Fig 5 *L bifidus* Subsurface colony in tomato agar × 125
Fig 6. *L. acidophilus* Subsurface colony in tomato agar × 48
Fig 7. *L bifidus* Surface colony on tomato agar × 25
Fig 8. *L acidophilus.* Surface colony on tomato agar. × 25

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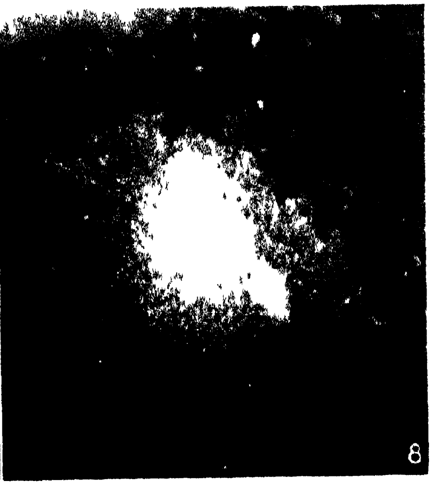

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(James L. Weiss and Leo F. Rettger: Lactobacillus bifidus)

THE PLEO-ANTIGENICITY OF A VARIANT OF PROTEUS X19

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The purpose of this paper is to report the finding of an unusual spontaneously occurring variant in a stock strain of Proteus X19. Because of the unusual biological reactions and because the organism presented a potential source of trouble in using the Weil-Felix reaction for laboratory diagnosis, we believe a detailed report is desirable.

The Weil-Felix reaction has been in use since Weil and Felix (1916) demonstrated that a culture of a proteus-like organism isolated from the urine of a patient with typhus fever would agglutinate to a high titer not only in the patient's serum but also in the sera of other patients suffering from typhus. Fletcher and Lessler (1926) later recorded a series of typhus cases in the Federated Malay States in which the patients' sera failed to agglutinate the classical X19 strain, but did agglutinate a variant strain. The variant (Kingsbury strain) differed from the typical Proteus X strains in failing to ferment maltose or sucrose, in not producing indol, and in being serologically distinct as tested by direct agglutination. Besides these cases, however, there were others occurring with equal frequency which gave the Weil-Felix reaction with the X19 strain.

Weil and Felix (1920) demonstrated that the agglutinogen of typhus fever virus was identical with the specific main receptor (O receptor) of Proteus X19 and hence that the agglutinins in typhus patients for Proteus X19 were due to a specific immunologic response. These results were confirmed and extended by Castaneda and Zia (1933) who concluded from their investigation there is a common antigenic factor in the Mexican Rickettsiae and Proteus X19 which explains the Weil-Felix reaction.

Studies by Weil (1920) demonstrated that variants of a Proteus X19 strain were serologically new organisms. In the same year Loewenhardt (1920) demonstrated that the sera of typhus fever patients frequently showed the presence of agglutinins for various kinds of bacteria particularly those of the typhoid-dysentery group. He believed that co-agglutination with typhoid was due to a previously formed antibody. This was not true in the case of dysentery. Recently an editorial in the Journal of the American Medical Association (1934) discussed the pleo-antigenicity of bacteria emphasizing the importance of this phenomenon to clinicians. The editor commented on the work of Fürth (1923) who demonstrated that some variants of the paratyphoid group gained antigenic components not present in the original parent culture.

The original culture used in this investigation was obtained ten years ago from The American Type Culture Collection. It was carried on standard "pneumo-agar" until September, 1932, at which time "Savita[1]-agar"—a yeast-extract, meat-free medium —was used. No change was noted in the original culture until one year later at which time it was noted that several Weil-Felix reactions were positive although there was nothing clinically which would indicate typhus fever. Control tests of supposedly normal sera were used and a high titer agglutination was found. A re-check of the supposedly pure stock strain revealed the presence of another organism and it was the attempt to explain this apparent dissociate which led to the following work.

In this investigation the authors have studied a variant of Proteus X19 with antigenic components that could not be demonstrated by similar direct agglutinations in the original parent culture. The variant agglutinated markedly with a variety of sera and on animal inoculation had the ability to produce agglutinins for the homologous organisms of these sera.

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[1] Extract of yeast. Battle Creek Food Company, Battle Creek, Mich.

To determine the relationship of the variant found to the Proteus X19 strain, studies were made of the morphological, physiological and serological characteristics of the parent strain and of its spontaneous variant. By growth in a Savita-serum broth using a pure culture of Proteus X19 as a parent strain and the homologous serum in varying dilutions, another variant was induced which had some characteristics in common with the variant originally found.

Throughout this article the pure strain of Proteus X19 found in the original culture will be referred to as the *parent strain*, the variant found in the original culture as *Variant A* and the laboratory-induced variant will be referred to as *Variant B*.

MORPHOLOGY

Microscopic. The parent strain and both its variants were similar microscopically. The organisms were fairly uniform, non-motile,[2] Gram-negative rods. Occasional filamentous forms were found in all three cultures although the variants appeared to be uniformly longer than the parent strain. Some of the filamentous forms showed Gram-positive granules.

Macroscopic. In general, the parent strain and its variants grew more readily on agar which had been standing long enough to lose its water of condensation and to present a very dry surface.

The parent strain showed a glistening, white growth on agar slants. This growth was slightly spreading with erose edges. In broth a fairly even turbidity was produced in twenty-four hours although a flocculent precipitate appeared in the bottom of each tube. On eosin-methylene blue agar the colonies, measuring 2 to 5 mm. in diameter, were raised and had rough edges and surfaces. On this medium the colonies had black centers indicating dye precipitation, and white, undulate peripheries.

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[2] Six months after completing the morphological studies, Variant B showed some motile forms. Both the Proteus X19 (parent strain) and Variant A strain were definitely non-motile in three, eight and twenty-four hour broth cultures.

On Krumwiede's triple sugar agar an acid and gas butt and an acid slant were produced.

On agar slants, Variant A produced a dull, raised, filiform growth. An even turbidity without sediment was produced in broth. On eosin-methylene blue agar the colonies, measuring 1 to 3 mm. in diameter, were raised, smooth, glistening and entire after forty-eight hours. No precipitation of the dye was observed. On Krumwiede's triple sugar agar an acid butt and an alkaline slant were produced.

Variant B grew very meagerly on agar at first but appeared to adapt itself to this medium with serial transplantation. On agar slants the growth was smooth, raised, glistening and filiform. On eosin-methylene blue agar the colonies, measuring 2 to 3 mm. in diameter, were smooth, raised, glistening and entire with discrete blue centers. In broth a slight turbidity, with a moderate amount of flocculent precipitate occurred after twenty-four hours. No change was produced in Krumwiede's triple sugar agar.

BIOCHEMICAL REACTIONS

The parent strain gave the typical biochemical reactions of the Proteus type. The reactions of both variants, characterized chiefly by failure to produce gas in carbohydrate media, were sufficiently distinct to differentiate them with ease from the parent strain. A summary of the reactions of each strain follows:

Parent strain. Acid and gas were produced in glucose, sucrose, salicin, maltose, raffinose, cellibiose, xylose and glycerol. No change was noted in lactose, mannitol, rhamnose, arabinose, inositol, dulcitol, sorbitol, galactose, adonitol and dextrin. The methyl red and nitrate tests were positive. The indol test was negative. The reaction in litmus milk varied from neutral to slightly alkaline. In lead acetate media H_2S was produced. Gelatin was liquefied.

Variant A. Acid was produced in glucose, lactose, maltose, mannitol, rhamnose, arabinose, raffinose, cellibiose, galactose, and glycerol. No change was noted in sucrose, salicin, inositol, dulcitol, xylose, sorbitol, adonitol and dextrin. The methyl red and nitrate tests were positive. Indol was not produced. Litmus milk was rendered slightly acid. In lead acetate media H_2S was produced. Gelatin was not liquefied.

Variant B. This organism grew poorly and even after eighteen days in carbohydrate media acid without gas was produced in only one sugar, glucose. The methyl red, nitrate, and indol tests were negative. H_2S was not produced in a lead acetate medium. Litmus milk showed no change. Gelatin was liquefied in twenty-four to forty-eight hours.

SEROLOGICAL STUDIES

After several transplantations and reisolations, Variant A was tested against diagnostic sera representative of the Salmonella, Eberthella and Shigella groups of organisms, all of which had been prepared during the past year. At the same time the parent strain and Variant B were tested against similar sera. All antigens used were prepared to correspond to a turbidity of 7.6 to 7.8 with the Gates' (1920) apparatus. The macroscopic tube test (1.0 ml. total volume) was used in all instances.

The parent strain agglutinated in an homologous serum but failed to agglutinate with representative sera of the Salmonella, Eberthella or Shigella groups of organisms. On the other hand Variant A not only showed a marked agglutination with the Proteus serum but also with the sera of *S. Schottmuelleri, S. paratyphi, S. enteritidis, S. Hirschfeldii, E. typhi, E. typhi O, Shigella dysenteriae, Shigella paradysenteriae* Sonne, Flexner and Army. This also was true to a lesser degree with Variant B. The marked antigenicity of these variants might suggest the possibility of spontaneous agglutination but in some of the sera used no agglutination occurred. Control tests showed no tendency toward spontaneous agglutination. In addition, work on agglutinin absorption reported below definitely showed that the agglutination was not of the spontaneous variety.

Since both Variant A and Variant B showed marked agglutination with several heterologous sera, the question arises as to whether these agglutinogens were acquired in the course of dissociation, or existed in the parent strain in such a state that they did not identify themselves by direct agglutination in heterologous sera. The parent strain showed no agglutination with similar sera. Without further study it would appear that the dissociates had acquired new antigenic components similar to those reported by Fürth (1923) for the paratyphoid group. Since the parent strain showed no agglutination in heterologous sera and since variants of this strain had this property, it appeared advisable to determine whether on injection into animals this parent strain could produce agglutinins corresponding to the agglutinogens shown to be present in the variants. Accordingly three rabbits were injected respectively with the original Proteus X19 culture (this culture contained both the parent and Variant A strains), the parent strain in pure culture and the Variant A strain in pure culture. These rabbits were injected every day with increasing doses of live organisms.

After ten injections the Proteus X19 culture (parent strain plus Variant A) as well as the parent strain in pure culture produced titers of about 1:2000. Ten injections of the Variant A in pure culture, however, failed to develop agglutinins for its homologous organism but agglutinins were present, at this time, for the parent strain. It was only after 18 injections that a low-titered Variant A serum was produced for its homologous organism. Even after 18 injections, however, the titer of the Variant A serum with the parent strain was greater than the titer of this serum for its homologous organism.

Because the original culture had present in it both the parent and Variant A strains, it seemed advisable to test only a short series of organisms in this serum, since no definite information could be obtained as to the source of any agglutinins found. Accordingly this serum was tested against its homologous culture, the parent strain (in pure culture), Variant A, *S. Schottmuelleri*, *E. typhi O* and *Shigella paradysenteriae* Sonne. Definite agglutination was obtained with the heterologous organisms in dilutions of 1:320 to 1:640.

The sera prepared for the parent strain (in pure culture) and for Variant A were tested against several heterologous organisms.

These organisms corresponded to the heterologous sera mentioned above. A summary of the reciprocal serological relationships of the parent and Variant A strains to heterologous organisms is given in table 1.

TABLE 1

Summary of results obtained with Proteus Variant A organism vs. heterologous sera and with Proteus X19 serum vs. corresponding heterologous organisms

HETEROLOGOUS SERA	AGGLUTINATION RESULTS				HETEROLOGOUS ORGANISMS
	Proteus variant A organism	Proteus X19 (parent strain) organism	Proteus variant A serum	Proteus X19 (parent strain) serum	
S. Schottmuelleri (Ufford)	+	0	0	0	*S Schottmuelleri* (Ufford)
S. paratyphi (Michigan)	+	0	+	+	*S. paratyphi* (Michigan)
S. Hirschfeldii	+	0	+	+	*S. Hirschfeldii*
S. enteritidis	+	0	+	+	*S. enteritidis*
E. typhi (Michigan)	+	0	+	+	*E. typhi* (Michigan)
E. typhi "O" (New York)	+	0	+	+	*E. typhi* "O" (New York)
Shigella dysenteriae Shiga	+	0	+	+	*Shigella dysenteriae* Shiga
Shigella paradysenteriae Sonne	+	0	+	+	*Shigella paradysenteriae* Sonne
Shigella paradysenteriae Flexner	+	0	+	+	*Shigella paradysenteriae* Flexner
Shigella paradysenteriae Army	+	0	+	+	*Shigella paradysenteriae* Army
Pseudomonas	0	0	0	0	*Pseudomonas*
Br. abortus (Mulford)	0	0	0	0	*Br. abortus* (Mulford)

Note: The titers of heterologous sera with Variant A varied between dilutions of 1:80 and 1:640; the titers of heterologous organisms with Variant A and *Proteus* X19 (parent strain) sera varied between dilutions of 1:80 and 1:320.

It will be noted (table 1) that both the parent and Variant A strains, on injection into animals, are capable of producing agglutinins for the members of the Salmonella, Eberthella and Shigella groups of organisms that were used with the single exception of *S. Schottmuelleri*. As previously noted, *S. Schottmuelleri* was agglutinated in the serum produced by injection of the parent strain plus Variant A. This was not the case with antisera produced by injection of pure cultures of either of these strains separately. However, since *S. Schottmuelleri* serum does agglutinate Variant A and Variant B but not the parent strain and since the Variant A serum has definitely a low titer, it would appear to us that this does not indicate the loss of an antigenic component of the Variant A strain but is probably due to the concentration of antibodies in the serum produced by this strain. Further studies on agglutinin absorption showed definitely antigenic components for *S. Schottmuelleri* in both the parent and Variant A strains.

Briefly summarizing the results obtained, we find that the parent strain alone does not agglutinate in heterologous sera. The variants of this parent strain both show a remarkable ability to agglutinate in a variety of heterologous sera. On injection of the parent strain or its variant into rabbits, agglutinins are produced which in general correspond to the agglutinogens shown for the variants. Apparently agglutinogens for a variety of sera are present in the parent strain but do not manifest themselves by direct agglutination of this strain in heterologous sera. These agglutinogens may be demonstrated either in the variants directly or in the parent strain by the production of antiserum. The ability of an organism to produce an agglutinin for another strain when it cannot be shown to agglutinate in the sera of such a strain has been demonstrated previously in the dysentery group of organisms by Welch and Mickle (1932).

Of the twelve heterologous sera used to agglutinate Variant A, all but the Pseudomonas and *Brucella abortus* sera showed ability to agglutinate this organism. Correspondingly, when Pseudomonas and *Brucella abortus* strains were tested against sera of the parent or the Variant A strains, negative results were obtained. Although *S. Schottmuelleri* serum was shown to agglutinate Variant A, neither Variant A nor the parent strain sera agglutinated *S. Schottmuelleri* (table 1). In view of these results we cannot consider that Variant A did acquire new antigenic components as was first thought to be the case early in this study except possibly in the case of *S. Schottmuelleri*. With this latter organism further study is necessary for definite proof.

AGGLUTININ ABSORPTION STUDIES

In order to establish more definitely the relationship of the parent Proteus X19 strain to Variant A and, further to explain the results obtained with the *S. Schottmuelleri* strain, a series of agglutinin absorption tests were made. Representative sera and organisms of the Salmonella, Shigella and Eberthella groups were used in this series.

Agglutinin absorption method. The agglutinating serum was diluted to a degree depending on its original titre. The dilutions varied from 1:10 to 1:50. Six milliliters of the diluted serum were added to a twenty-four-hour agar slant of the organism under study. With a platinum loop, the suspension was carefully emulsified in the serum. A small pledget of sterile absorbent cotton was dropped into the test tube and the suspension drawn up into a pipette through the cotton which acted as a filter for gross particles. The suspension was pipetted directly to a sterile centrifuge tube, incubated in a water bath at 37°C. for two hours, and then centrifuged at high speed for one hour or until the supernatant fluid was clear. (The cotton plugs were fixed firmly in the centrifuge tubes with elastic bands.) The supernatant fluid was then pipetted under sterile conditions onto a second twenty-four-hour slant culture and the procedure repeated. The procedure was then repeated on a third slant. (This third suspension may be placed in the icebox over night with equally good results.) Absorption with homologous organisms using this technique is invariably complete.

Results. The results obtained by agglutinin absorption are given in table 2. The parent strain serum was absorbed with Variant A, *E. typhi* "O" (New York) and *S. Schottmuelleri*, respectively. The Variant A serum was absorbed with the parent strain, *E. typhi* "O" (New York) and *S. Schottmuelleri*. The *S. Schottmuelleri* serum was absorbed with Variant A and with the parent strain. The sera of *S. enteritidis*, *E. typhi* (Michigan) and *Shigella paradysenteriae*, both Sonne and Army varieties, were absorbed with the parent strain. Duplicate unabsorbed control sera were titrated at the same time these absorptions were made.

It will be noted in table 2 that both unabsorbed parent strain and Variant A sera agglutinated all organisms used except *S.*

TABLE 2

Agglutinin absorption tests

UNABSORBED PROTEUS X19 (PARENT STRAIN) SERUM	ORGANISMS	ABSORBED PROTEUS X19 (PARENT STRAIN) SERUM WITH:		
		Variant A	*E. typhi* "O"	*S. Schottmuelleri*
1280	*Proteus* X19 (parent strain)	1280	1280	1280
640–1280	Variant A, *S. enteritidis*, *E. typhi*, *Shigella paradysenteriae* Sonne	0	0	0
160–320	*S. paratyphi*, *S. Hirschfeldii*, *E. typhi* "O," *Shigella dysenteriae*, *Shigella paradysenteriae*, Army, Flexner			
0	*S. Schottmuelleri*			

UNABSORBED VARIANT A SERUM	ORGANISMS	ABSORBED VARIANT A SERUM WITH:		
		Proteus X19 (parent strain)	*E typhi* "O"	*S. Schottmuelleri*
640	*Proteus* X19 (parent strain)	0	640	1280
	Variant A	0	0	0
320	*S. paratyphi*, *S. enteritidis*, *S. Hirschfeldii*, *E. typhi*, *E. typhi* "O," *Shigella dysenteriae*, *Shigella paradysenteriae*, Sonne, Army, Flexner			
0	*S. Schottmuelleri*			

UNABSORBED SERA	TITRES			ABSORBED WITH:	TITRES		
	Homologous organism	Proteus X19 (parent strain)	Variant A		Homologous organism	Proteus X19 (parent strain)	Variant A
S. Schottmuelleri	2560	0	320	Proteus X19 (parent strain)	2560	0	0
				Variant A	2560	0	0
S. enteritidis	1280	0	640	Proteus X19 (parent strain)	1280	0	0
E. typhi	1280	0	640		1280	0	0
Shigella paradysenteriae Sonne	1280	0	320		640	0	0
Shigella paradysenteriae Army	2560	0	320		1280	0	0

Note: Table shows final significant titers of the unabsorbed and absorbed sera used.

Schottmuelleri even though the serum produced by the injection of these organisms (parent strain and Variant A) together contains agglutinins for this strain (*S. Schottmuelleri*). Since, also, the *S. Schottmuelleri* serum itself agglutinates Variant A but not the parent strain it would appear, as noted previously, that this lack of ability of the parent strain and Variant A sera to agglutinate the *S. Schottmuelleri* strain is due to a weakness of these sera rather than a lack of antigenicity inherent in the parent or Variant A strains. This is borne out when *S. Schottmuelleri* serum is absorbed with either the parent or Variant A strains. These absorptions show definitely that both the parent and Variant A strains have an antigenic component in common with *S. Schottmuelleri* since the absorption of *S. Schottmuelleri* serum with the parent strain removes agglutinins for Variant A. In this connection it has already been shown that Variant A agglutinates in *S. Schottmuelleri* serum (table 1).

The absorption of the parent strain serum with Variant A organism removed all but the homologous agglutinins of this serum. The absorption of the parent strain serum with *E. typhi* "O" or with *S. Schottmuelleri* gave identical results; i.e., the agglutinins present in this serum for Variant A, *S. paratyphi* (Michigan), *S. Hirschfeldii*, *S. enteritidis*, *E. typhi* (Michigan), *E. typhi* "O" (New York), *Shigella dysenteriae* and for *Shigella paradysenteriae* Sonne, Army and Flexner varieties were completely removed.

The absorption of Variant A serum with the parent strain removed all agglutinins including the homologous agglutinins from this serum. The absorption of Variant A serum with *E. typhi* "O" or *S. Schottmuelleri* removed all agglutinins except those present in this serum for the parent strain. These results correspond to those obtained when the parent strain serum was similarly absorbed. It would appear then that the heterologous agglutinins found in the Proteus X19 (parent strain) and Variant A sera are group agglutinins.

Since a variety of heterologous sera were shown to agglutinate the Variant A strain but *not* the parent strain, it seemed advisable to absorb certain of these sera with the parent strain in order to determine whether this organism, although unable to agglutinate in these sera, could remove from them the agglutinins present for its variant. The results of these absorptions with controls are given in table 2. All the sera were absorbed with the parent strain. The serum of *S. Schottmuelleri* was also absorbed with Variant A. (This latter absorption, run as a control, removed completely the Variant A agglutinins.) In each case where a serum, known to agglutinate Variant A, was absorbed with the parent strain, the agglutinins present in such sera for Variant A were removed in spite of the fact that the absorbing organism did not have the ability *per se* to agglutinate in these sera.

HETEROPHILE ANTIGEN STUDIES

In view of the fact that a number of heterologous sera were shown to be capable of agglutinating variants of a Proteus X19 strain in contrast to a corresponding lack of ability to produce a similar reaction with the parent strain, it seemed possible that these results might be explained on the basis of the presence of a heterophile antigen in the variant that was absent in the parent Proteus X19 strain. In favor of this possibility is the investigation of Abe (1924) who demonstrated that *Proteus vulgaris* X19 lacked the heterophile or Forssman antigen.

Method. Sera were tested for heterophile antibody (hemolysin) by diluting with sterile isotonic salt solution 1:4 through 1:1024. Guinea pig complement used in the regular Wassermann test and titrated daily was added in the proper dilution in 0.4 ml. amounts to 0.5 ml. of the serum dilutions. (The complement dilution varied from 1:10 to 1:12.) Washed (5 per cent) sheep cells (0.1 ml.) were then added giving a final concentration of 0.5 per cent sheep cells and making a total volume of 1.0 ml. The tubes were shaken and incubated in the 37°C. water bath for one hour and read. Appropriate controls were incubated at the same time.

The organisms under study were tested for heterophile antigen by direct agglutination in a known heterophile antiserum. The heterophile serum[3] was prepared by the injection of guinea pig kidney emulsion into rabbits and had a titer of approximately 1:40,000. The bacterial antigens had a turbidity corresponding to 7.6 to 7.8 on the Gates (1920) apparatus. Dilutions were made from 1:100 to 1:12,800 in a regular sequence. The total volume in each tube was 1.0 ml.

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[3] We are indebted to Dr. C. A. Stuart, Brown University, Providence, R. I., who furnished this serum.

Results. On testing the Proteus X19 (parent strain), Proteus Variant A, *E. typhi*, *E. typhi* "O," *S. Schottmuelleri*, *S. enteritidis*, *Shigella paradysenteriae* Sonne and Army sera for presence of heterophile antibody (hemolysin) it was found that all sera (including parent-strain serum) tested except Variant A serum showed the presence of a heterophile antibody, the titers varying from 1:32 to 1:512 (0.5 per cent sheep cell suspensions final concentrations were used). The presence of heterophile antibody in the parent strain serum obviates the possibility of explaining the contrasting results obtained in agglutinating the parent strain and Variant A in heterologous sera. However, the apparent absence of heterophile antibody in Variant A serum does not necessarily demonstrate the lack of heterophile antigen in the organism used to produce this serum. Because of the difficulty encountered in producing the Variant A serum it seemed advisable to test the homologous organisms of the sera mentioned above for heterophile antigen. (Later work, using more dilute sheep cell suspensions, demonstrated the presence of small amounts of heterophile hemolysin but not heterophile agglutinin in Variant A serum.)

On testing Proteus X19 (parent strain), Proteus Variant A, *E. typhi*, *E. typhi* "O," *S. Schottmuelleri*, *S. enteritidis*, *Shigella paradysenteriae* varieties Sonne and Army for the presence of heterophile antigen using agglutination in a known heterophile serum as an index (homologous titer 1:40,000) titers of 1:400 to 1:800 were obtained in all instances with the single exception of Proteus X 19 (parent strain). No heterophile antigen was demonstrated in the parent strain even though the serum of this organism contained heterophile hemolysin. The reaction of the parent strain in heterophile serum is similar to its reaction in heterologous sera, i.e., it fails to agglutinate in such sera but produces agglutinins on animal injection.

If we correlate the above results with those obtained previously (where it was shown that Variant A would agglutinate in a series of heterologous sera), it would appear that these agglutinations of Variant A might be due to the presence of heterophile antibody in heterologous sera. The presence of heterophile antibody in the serum of the parent strain might also explain the agglutination of heterologous organisms with this serum.

In order to determine whether the agglutination of Variant A by heterologous sera and the agglutination of heterologous organisms with the parent strain serum were due to heterophile relationships, or to definite bacterial antigenic components of the parent and Variant A strains, the heterologous sera used throughout this study were absorbed with sheep cells to remove the heterophile antibody and titrated, after absorption, against Variant A. Assuming that the agglutination of Variant A by heterologous sera was due to the presence of heterophile antibody, the absorption of these sera with sheep cells would remove the Variant A antibodies.

In view of the fact that the agglutination of the Variant A strain might possibly be due not to heterophile agglutinin but to isophile agglutinin, it appeared advisable also to absorb the heterologous sera with boiled sheep cells, since it is known that boiling sheep cells for thirty minutes destroys the isophile antigen.

Method. The method used for the absorption of sera with sheep cells was similar to the method used for the absorption of sera with bacteria reported elsewhere in this paper. Each serum was absorbed three times with washed sheep cells using 0.5 ml. of packed cells in each instance. (The packed sheep cells were prepared from a 5 per cent suspension in isotonic salt solution.) The sera were decanted onto the packed sheep cells in sterile centrifuged tubes and the cells resuspended. The centrifuge tubes were then placed in a 37°C. water bath for two hours. The sheep cells were then packed again in the centrifuge (fifteen minutes at 1500 r.p.m.) and the sera decanted into a fresh centrifuge tube containing 0.5 ml. of packed sheep cells. This process was repeated twice similarly to the absorption with bacteria. The absorption of heterophile antibody using the above method was complete in each instance. The same method was used in the absorption of sera with sheep cells from which the isophile antigen had been removed by boiling.

All absorbed sera were tested for both heterophile agglutinin and heterophile hemolysin since we found the latter test more sensitive. Unabsorbed control sera and sera absorbed with boiled sheep cells were titrated[4] at the same time. It was found that with each serum after absorption with sheep cells, the heterophile antibody was completely removed but that these absorbed sera continued to agglutinate Variant A undiminished in some degree. Whether the sera studied were absorbed with fresh or with boiled sheep cells the same results were obtained indicating that we were dealing with heterophile and not isophile antibody in these sera. It would appear from these results that the agglutination of Variant A by heterologous sera and the agglutination of heterologous organisms in the parent strain serum is not due to heterophile relationships but to the pleo-antigenicity of these Proteus strains.

DISCUSSION

The importance to diagnostic laboratories in the public health field of the finding of pleo-antigenic variants of Proteus X19 in stock cultures, when such cultures are used daily in the diagnosis of typhus fever, is obvious. The particular culture studied in this investigation, containing a variant of a Proteus X19 strain, was discovered when it was found that the original culture used in the Weil-Felix reaction was giving positive results on patients with no symptoms of typhus fever. On further study, the culture used for diagnosis was found to be made up of two strains—a true Proteus X19 and a variant strain. It was further shown that the variant strain was responsible for the false positive results obtained and that the Proteus X19 strain, when used alone, agglutinated only in its homologous serum and did not give false positive results in the Weil-Felix reaction. The Proteus X19 strain apparently dissociated spontaneously after being carried over a long period of time on artificial media. Since it was found possible to induce in the laboratory another variant of the parent Proteus X19 strain in some respects similar to the variant that appeared spontaneously in this stock culture, it is reasonable to expect that other diagnostic laboratories may have to deal with a similar situation.

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[4] Instead of using 0.5 per cent sheep cells for the hemolysin test a 0.02 per cent suspension was used. Small amounts of heterophile hemolysin were demonstrated in this case for unabsorbed Variant A serum in contrast to the results obtained previously when using the 0.5 per cent suspensions.

The marked pleo-antigenicity of the variants of the Proteus X19 strain studied appeared at first to be an example of the acquisition of new antigenic components since the variants would agglutinate with a variety of heterologous sera (*dysentery*, *typhoid*, *paratyphoid* and *enteritidis*) whereas the parent strain agglutinated only in its homologous serum. However, further study of the sera of the parent and Variant A strains indicated that this was not the case. The parent strain produced agglutinins for several heterologous organisms. It would seem that this is not a demonstration of acquired antigenic components but that the parent strain does have in its make-up antigenic components for the heterologous sera used. It would appear to us of fundamental significance that the dissociation of an organism brings about a "freeing" or rearrangement of agglutinogens of the induced variant so that they can be demonstrated in an agglutination test where previously such results were not demonstrable. For example, when the Proteus X19 organism is dissociated, the antigenic structure of the induced variant strain is such that direct agglutination in heterologous sera becomes possible. A similar freeing of agglutinogens is obtained on animal injection of the parent strain resulting probably from hydrolytic cleavage of the bacterial antigen, in the sense of Manwaring (1928), since heterologous agglutinins are found in the serum produced.

A definite antigenic relationship between the parent Proteus X19 strain and its variant was demonstrated by agglutinin absorption. On absorbing heterologous sera, which were capable of agglutinating the variant strain but not the parent strain, with Proteus X19, the agglutinins for the variant strain were removed from these sera.

Heterophile antigen was demonstrated in both Proteus strains —in the parent strain by demonstration of heterophile hemolysin in the serum produced by this organism—in Variant A by a similar observation, as well as by demonstration of a heterophile agglutinogen in the antigenic structure of this organism by agglutination in a heterophile serum. The marked pleo-antigenicity of these Proteus strains was demonstrated to be due to bacterial group agglutinogens and not solely, if at all, to the presence in these strains of heterophile antigen.

Further studies of Proteus X19 and Proteus Variant strains obtained from various sections of this country and Canada are now in progress.

SUMMARY AND CONCLUSIONS

1. A pleo-antigenic variant of a Proteus X19 strain was shown to be the cause of false positive reactions in the Weil-Felix test.
2. The Proteus X19 strain, although inagglutinable in certain heterologous sera, was shown, by agglutinin absorption, to have agglutinogens in common with the organisms used to produce these sera.
3. The heterologous agglutinogens, shown in the variant strain by direct agglutination, were not acquired in the process of dissociation but were present in the parent strain although not demonstrable by the same method.
4. The heterologous relationships of the Proteus X19 strain and its variant were shown to be due to bacterial group agglutinogens and not soley, if at all, to the presence of heterophile antigen in these strains.

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OXIDATION-REDUCTION POTENTIALS AND FERRICYANIDE REDUCING ACTIVITIES IN PEPTONE CULTURES AND SUSPENSIONS OF ESCHERICHIA COLI

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Oxidation-reduction potentials developed in cultures of *Esch. coli* have been reported by Cannan, Cohen, and Clark (1928), Hewitt (1931) and Boyd and Reed (1931). Boyd and Reed presented evidence that both gaseous (hydrogen) and non-gaseous electromotively active systems are responsible for the potentials observed at different periods of growth in anaerobic cultures of *Esch. coli*. The studies to be reported here were confined to the potentials developed in stationary and continuous flow cultures of *Esch. coli* in peptone, a somewhat less complex medium than those employed by other investigators.

It has been reported (Clifton, 1933a) that broth cultures of *Staphylococcus aureus* in or near the maximum stationary growth phase reduce potassium ferricyanide at a rate in the neighborhood of 6.6×10^{-14} millimols per cell per minute. These studies have been extended to the rates of reduction of ferricyanide at different stages of growth in stationary and continuous flow cultures of *Esch. coli*. Studies on the influence of the concentration of peptone, ferricyanide and organisms on the rate of reduction of ferricyanide in suspensions of "resting" *Esch. coli*, as outlined in a preliminary paper by one of us (Clifton, 1933b), are also presented.

METHODS

Three-necked pyrex distilling flasks of 500 ml. capacity were employed as culture vessels. Two platinum electrodes, sealed in soft glass tubing and supported by a rubber stopper, were inserted through the central neck of the flask. A glass tube, extending to the bottom of the flask, was also supported by this stopper. This tube, well plugged with cotton at the upper end, served as an inlet for the compressed air or nitrogen employed in stirring the cultures before samples were removed for pH determinations, for plate counts and for ferricyanide reduction tests. This procedure ensured a thorough mixing of the contents and a greater uniformity in sampling. The side necks of the flasks were plugged with cotton and the system, containing 300 ml. of the culture medium, was sterilized in the autoclave. This half-cell was connected by means of a sterile saturated potassium chloride-agar bridge, supported by a cotton plug in one side neck of the flask, to the standard saturated potassium chloride-calomel electrode.

A similar system (fig. 1) was employed for the continuous flow cultures with the addition of an automatic, constant level siphon supported by the central stopper and a piece of thermometer tubing passing through a stopper in an extension of the side neck opposite the salt bridge. This capillary tube was connected to a peptone supply flask supported above the culture chamber and served to regulate the rate of flow of the solution.

Representative samples of the continuous flow cultures were obtained in the following manner: (1) the outlet tube attached to the small reservoir at the end of the automatic siphon was closed by means of a pinch-cock; (2) the siphon column was broken; (3) the culture was vigorously stirred with air for ten minutes; (4) the siphon was again placed in operation, and (5) a sample was immediately drawn over into the small reservoir at the end of the siphon. The removal of the sample from this receptacle, by means of a pipette, inserted through the sampling tube, reduced the possibilities of contaminating the culture.

The ferricyanide reduction tests were carried out in Pyrex tubes (3 × 20 cm.) to which were attached two side arms. One arm, attached to the bottom of the tube and running to the top where it was bent at a right angle, served as an inlet for the purified nitrogen employed in stirring the sample and maintaining anaerobic conditions. The other arm, attached near the top of the tube, served as an outlet for the gas which escaped under water in a second tube. A platinum electrode and salt bridge were supported by a rubber stopper in the top of the reduction test tube. An opening, normally closed by a glass rod, in this stopper permitted the introduction of the ferricyanide solution after deaeration of the sample.

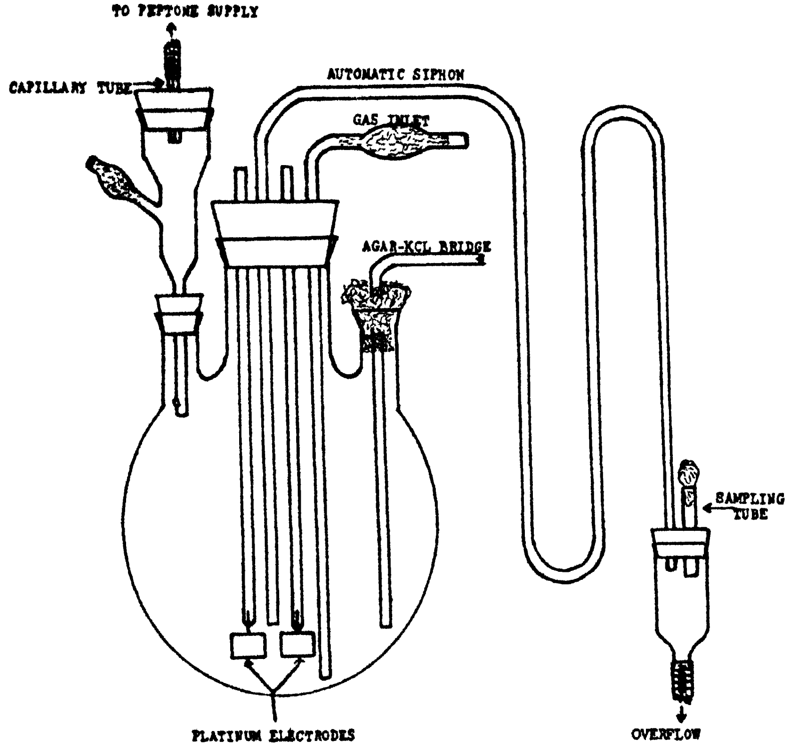

Fig. 1. Culture Chamber for Continuous Flow Cultures

Solutions of Difco peptone in 0.5 per cent saline or in an equivalent concentration of salt plus La Motte's phosphate buffers were employed in these studies. Freshly prepared solutions containing 3 parts of 0.1 molar Baker's C.P. $K_3Fe(CN)_6$ and 1 part of 0.1 molar $K_4Fe(CN)_6 \cdot 3H_2O$ were employed in the ferricyanide reduction tests, the final concentration being 0.01 molar. The E_0 of a 0.01 molar ferri-ferrocyanide system in a 1.0 per cent peptone solution at 37.5°C. was +0.391 volt. The concentration of ferricyanide at any time was determined directly from the potential readings. In the majority of the tests the rate of reduction was calculated from the time required to reduce the ferricyanide from a concentration of 60 per cent to a concentration of 40 per cent. During this time the rate of decrease in ferricyanide concentration with time was quite constant.

The ferri-ferrocyanide system was employed almost exclusively as the oxidation-reduction indicator because (1) it reacts very rapidly with the activated substrate; (2) it does not attack readily other substances in the reaction mixture; (3) it is a readily reversible and highly soluble electromotively active system characterized by a high E_0 practically independent of the pH under the conditions of these experiments; (4) the reduced form is not readily oxidized by traces of oxygen, and (5) in general it is better adapted to studies of this nature than the reversible dyestuffs commonly employed in other studies.

Viable counts were made by a standardized dilution and plating technic. All plates were incubated at 37.5°C. for forty-eight hours. The pH determinations were made with a Leeds and Northrop glass electrode assembly at room temperature and were checked at times against quinhydrone electrode and colorimetric determinations. All values reported are rounded off to the nearest 0.1 pH unit.

The time-potential and ferricyanide reduction tests were carried out in an oil bath at 37.5° ± 0.1°C.

EXPERIMENTAL RESULTS

Stationary aerobic cultures

Under aerobic conditions the potential of a 1.0 per cent Difco peptone, 0.5 per cent sodium chloride solution, pH 7.0 was poorly poised in the neighborhood of +0.250 volt. Deaeration of this system with a stream of nitrogen resulted in a negative drift in potential to a value near +0.125 volt.

When 300 ml. of this medium were inoculated with 0.5 ml. of an eighteen-hour peptone culture of *Esch. coli* (laboratory strain K-12) a rapid drop in potential occurred simultaneously with the period of rapid growth of the organisms and a maximum reducing potential of −0.150 volt was established between the twenty-fourth and thirty-sixth hour. This maximum reduction potential was 270 mv. more negative than the potential observed in sterile deaerated peptone media and about 250 mv. more negative than the potential observed in a deaerated L 3 Chamberland filtrate of a twenty-four hour culture. Typical time-potential, time-population and time-pH relationships are presented in figure 2.

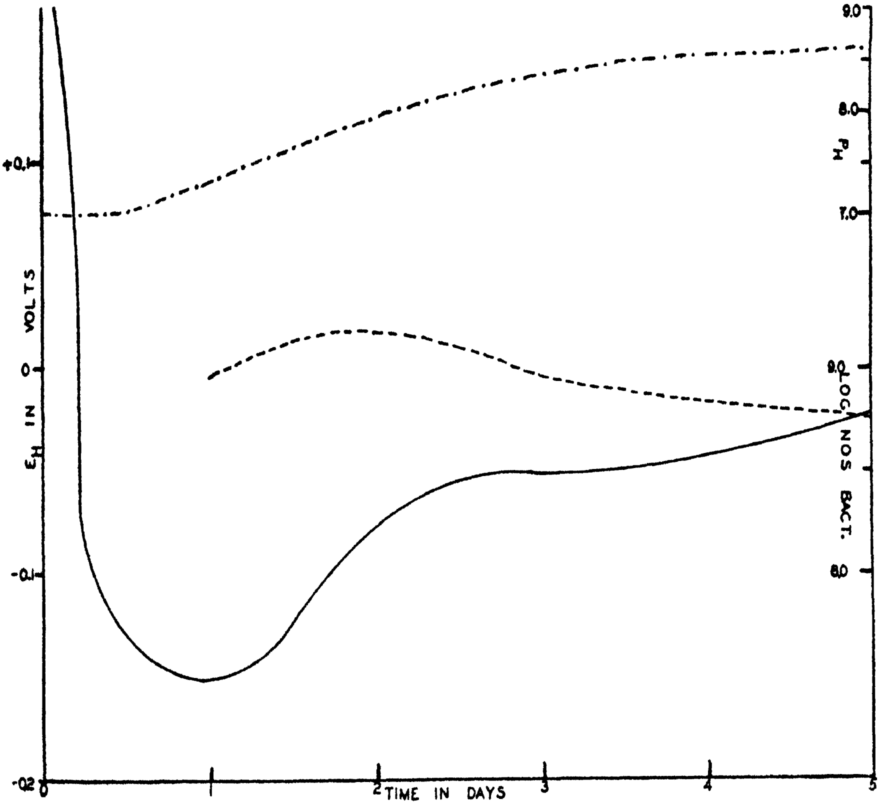

Fig. 2. Time-potential (—), Time-population (— — —), and Time-pH Relationships (.–.–.–) in an Aerobic Peptone Culture of *Esch. coli*

In similar tests the cultures were vigorously stirred with *air* for ten minutes to ensure thorough mixing before samples were removed for plate counts, pH determinations, and ferricyanide reduction tests. During the period of aeration of the cultures a rapid positive shift in potential of 150 to 200 millivolts was observed but on standing the potential returned, somewhat less rapidly, to the original level. Stirring the cultures with nitrogen had little effect on the observed potentials but tended to establish somewhat closer agreement between individual electrodes.

To retard the marked shift in hydrogen ion concentration observed in these cultures, pH 7.0 to 8.6 by the fifth day, a 1 per cent peptone solution was weakly buffered at pH 7.0 with phosphates to a final concentration of M/50. The maximum reducing potential, −0.130 volt, was developed when the culture was about twenty hours old. During the next twenty-four hours the potential shifted to −0.110 volt (pH 7.4) and was maintained at this level until the culture was discarded on the sixth day (pH 8.0).

A time-potential relationship similar to that observed with 1 per cent peptone cultures was noted when the peptone concentration was increased to 2.5 per cent. A maximum reduction potential of −0.195 volt and a maximum viable population of 110×10^7 per milliliter were developed on the second day.

Nine milliliter samples of the cultures were placed in the reduction tubes and deaerated with nitrogen for one-half hour before the addition of 1 ml. of a freshly prepared 0.075 molar potassium ferricyanide, 0.025 molar potassium ferrocyanide solution. The potential immediately shifted to the E_h level of this ferri-ferrocyanide system and then decreased with time in a manner similar to that reported for cultures of *Staphylococcus aureus*.

The rate of fall in potential ordinarily reached a steady value within twenty to thirty minutes and was of the order of 0.3 mv. per minute in a typical twenty-four hour, 1 per cent peptone culture of *Esch. coli*. Duplicate tests agreed within 2 per cent of each other. The above rate of fall in potential is equivalent to a total reduction of 0.00028 millimol of ferricyanide per minute. The total amount of ferricyanide reduced per minute decreased as the age of the cultures increased and in cultures older than five to seven days was of such a low magnitude that the rate of change in potential was ordinarily less than 0.04 mv. per minute. Since a sterile solution of peptone reduces ferricyanide at a rate in the neighborhood of 0.02 mv. per minute the results of the ferricyanide reduction tests obtained with the older cultures are only suggestive.

Plate counts carried out before and after the ferricyanide reduction tests indicated that these reagents were not discernibly toxic in the concentration employed during the course of these tests which rarely lasted more than two hours. However, this concentration (total = 0.01 M) of potassium ferricyanide and potassium ferrocyanide does retard growth to some extent in young cultures. The results of the reduction tests with samples from a typical culture are recorded in table 1, together with the averages of the results obtained with 6 similar cultures.

TABLE 1

Rates of reduction of $K_3Fe(CN)_6$ by 1 per cent peptone cultures of Esch. coli

AGE OF CULTURE	PLATE COUNT PER MILLILITER × 10^7	MILLIMOLS $K_3Fe(CN)_6$ REDUCED		AVERAGE RATE OF REDUCTION PER CELL PER MINUTE (6 CULTURES)
		Per minute	Per cell per minute	
hours				
24	90	0.00028	3.45×10^{-14}	3.06×10^{-14}
48	146	0.00010	0.76×10^{-14}	1.61×10^{-14}
72	90	0.00009	1.11×10^{-14}	1.18×10^{-14}
96	76	0.00013	1.93×10^{-14}	1.95×10^{-14}
120	60	0.00005	0.92×10^{-14}	1.00×10^{-14}

In the majority of the tests the highest rate of reduction per cell per minute was observed in twenty-four-hour cultures. This rate of reduction tends to decrease as the age of the culture increases, although individual variations were observed, particularly around the fourth day.

During the period of rapid growth the rate of reduction of ferricyanide (0.001 M mixture), as calculated from Buchanan's (1930) formula, ranged from 2.45×10^{-13} to 5.62×10^{-14} millimols per cell per minute.

A behavior similar to that observed with 1.0 per cent peptone cultures was noted when the peptone concentration was increased to 2.5 per cent. However, the rate of reduction in the 2.5 per cent peptone culture was greater than that observed in the 1 per cent peptone cultures. Typical results are given in table 2.

In another experiment a 1.0 per cent peptone solution in equal amounts of M/15 phosphates (pH 7.4) and 0.5 per cent saline solutions was employed as the culture medium. After twenty-four hours growth at 37.5°C. the culture reduced ferricyanide at a rate of 4.44×10^{-14} millimols per cell per minute. When a 1:1 dilution of the ferri-ferrocyanide mixture was employed the rate of reduction decreased to 3.84×10^{-14} millimols per cell per minute. When the concentration of cells was reduced to one-half the original value by dilution with saline the rate of reduction was 4.03×10^{-14} millimols per cell per minute. The addition of 1.0 ml. of a 10 per cent peptone solution to a sample similar to the above increased the rate of reduction to 9.09×10^{-14} millimols per cell per minute. Similar results were obtained with older cultures and are summarized in table 3.

TABLE 2

Rates of reduction of $K_3Fe(CN)_6$ in 2.5 per cent peptone cultures of Esch. coli

AGE OF CULTURE	PLATE COUNT PER MILLILITER × 10^7	MILLIMOLS $K_3Fe(CN)_6$ REDUCED	
		Per minute	Per cell per minute
hours			
24	80	0.00053	7.36×10^{-14}
48	110	0.00051	5.15×10^{-14}
72	83	0.00036	4.82×10^{-14}
96	73	0.00029	4.41×10^{-14}
144	51	0.00018	3.92×10^{-14}

These and similar results show that small dilutions of the cultures ordinarily increased to a slight extent the rate of reduction of ferricyanide. The same behavior was noted in suspensions of "resting" bacteria. The addition of fresh peptone to the samples markedly increased the rate of reduction. In the above test when 0.1 ml. of 10 per cent peptone was added to 9.0 ml. of the culture the increase in peptone concentration was only from 1.0 to 1.1 per cent but the rate of reduction increased nearly ninefold. This suggests that there may be a small fraction of the peptone that is most readily assimilated by the cells.

A sample of this buffered twenty-four-hour culture was tested for its reducing activity after twenty-four and forty-eight hours incubation at 3 to 5°C. The rate of reduction of the original twenty-four-hour culture was 4.44×10^{-14} and after twenty-four and forty-eight hours in the refrigerator was 4.61 and 4.06×10^{-14} millimols per cell per minute, respectively. The plate counts were 55, 53, and 52×10^7 per ml.

TABLE 3

The effect of dilution and of peptone concentration on the rate of reduction of $K_3Fe(CN)_6$ by a buffered (M/30 phosphates, pH 7.4) culture of Esch. coli

AGE OF CULTURE	pH OF CULTURE	PLATE COUNT PER MILLILITER	10 PER CENT PEPTONE ADDED	MILLIMOLS $K_3Fe(CN)_6$ REDUCED PER CELL PER MINUTE IN	
				9 ml culture	4.5 ml. culture
hours			*cc*		
24	7.5	55×10^7		4.44×10^{-14}	4.03×10^{-4}
			1.0		9.09×10^{-4}
48	7.65	128×10^7		0.96×10^{-14}	1.56×10^{-4}
			0.5		11.98×10^{-4}
			1.0		17.36×10^{-4}
72	7.8	130×10^7		0.68×10^{-14}	1.02×10^{-4}
			0.1	5.64×10^{-14}	
			1.0		16.24×10^{-4}

CONTINUOUS FLOW CULTURES

The automatic siphons in the continuous flow culture flasks were adjusted to retain 250 ml. of the 1.0 per cent peptone solution in the flasks and capillary tubes were selected to give a daily flow of approximately 500 and 250 ml. in experiments CF 1 and CF 2, respectively.

During the first twenty-four hours of growth in the continuous flow cultures, seeded with 0.5 ml. of an eighteen-hour peptone culture of *Esch. coli*, the potential decreased approximately 400 mv. A slow negative potential drift continued for eight to ten days and reached a level 50 to 75 mv. more negative than the maximum reduction potentials developed in the stationary (typical aerobic) cultures. The E_h was maintained at this low level until the cultures were discarded on the twenty-fourth day.

A maximum viable population was established within five to eight days. The value for the viable count dropped slowly after the tenth day and concomitantly the rates of flow of the peptone solutions decreased due to the accumulation of suspended matter in the capillaries. The peptone supply flasks were disconnected on the twenty-fourth day at which time the rates of flow had decreased to less than one-half of the original values.

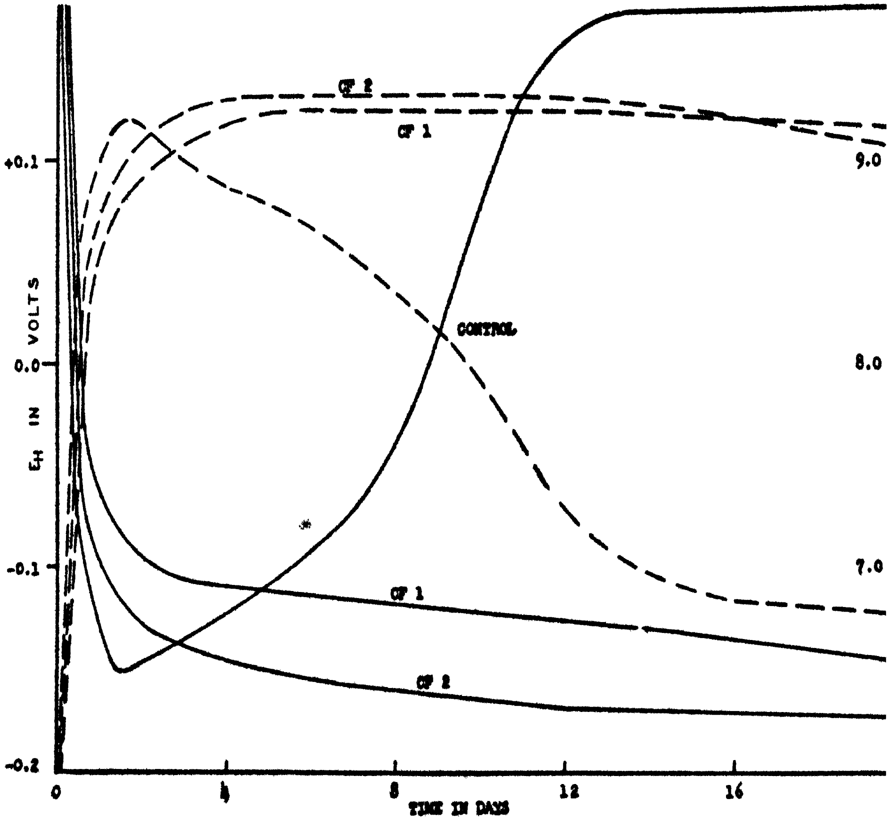

FIG. 3. TIME-POTENTIAL (—) AND TIME-POPULATION RELATIONSHIPS (— —) IN CONTINUOUS FLOW PEPTONE CULTURES OF *Esch. coli*

CF 1, 500 ml.; CF 2, 250 ml. daily flow and in a stationary culture (control).

The potentials and counts then shifted in a manner similar to those observed in the stationary (ordinary aerobic) cultures after maximum populations had been established. In these continuous flow cultures the pH remained quite constant in the neighborhood of 7.6. A similar behavior was observed in eight separate tests and in one test with 2.5 per cent peptone. The time-potential and time-population relationships for the continuous flow cultures 1 and 2 are graphically recorded in figure 3.

TABLE 4

Rate of reduction of $K_3Fe(CN)_6$ in constant flow cultures of Esch. coli

CULTURE	AGE	PLATE COUNT PER MILLILITER × 10^7	MILLIMOLS $K_3Fe(CN)_6$ REDUCED	
			Per minute	Per cell per minute
	hours			
1.0 per cent peptone (500 ml. daily)	24	66	0.00016	2.62 × 10^{-14}
	48	87	0.00045	5.48 × 10^{-14}
	72	94	0.00042	4.93 × 10^{-14}
	120	170	0.00059	3.84 × 10^{-14}
	168	168	0.00054	3.57 × 10^{-14}
	240	162	0.00044	3.05 × 10^{-14}
	460	129	0.00044	3.74 × 10^{-14}
	576	142	0.00071	5.58 × 10^{-14}
1.0 per cent peptone (250 ml. daily)	24	79	0.00024	3.40 × 10^{-14}
	48	115	0.00041	3.94 × 10^{-14}
	72	130	0.00034	2.89 × 10^{-14}
	120	190	0.00042	2.43 × 10^{-14}
	168	190	0.00040	2.34 × 10^{-14}
	240	164	0.00037	2.51 × 10^{-14}
	460	102	0.00050	5.44 × 10^{-14}
	576	88	0.00016	2.03 × 10^{-14}
2.5 per cent peptone (250 ml. daily)	24	50	0.00040	8.88 × 10^{-14}
	48	132	0.00060	5.05 × 10^{-14}
	96	134	0.00057	4.71 × 10^{-14}
	120	138	0.00096	7.73 × 10^{-14}
	144	150	0.00099	7.33 × 10^{-14}

Ferricyanide reduction tests with the continuous flow cultures were carried out in the manner previously described on 9 ml. samples drawn from the body of the well stirred cultures. Typical results are given in table 4.

These results show a general tendency on the part of the continuous flow cultures to maintain a higher rate of reduction per cell per minute than was observed with the stationary cultures. Also a higher rate of reduction was observed when either the peptone concentration or the rate of flow of the peptone was increased.

In a continuous flow 1.0 per cent peptone culture, 0.0075 and 0.0025 molar with respect to potassium ferricyanide and potassium ferrocyanide, respectively, the E_h slowly fell from an initial value of +0.421 volt to +0.312 volt on the fourth day at which time the viable population was only 19×10^7 per milliliter. This concentration of potassium ferri- and ferrocyanide was apparently slightly toxic to the actively growing cells. In a constant flow culture containing only one-tenth of the above concentration of ferri- and ferrocyanides the potential slowly fell through the range of this system during the early stages of growth and reached an E_h level of +0.035 volt and a population of 176×10^7 viable cells per milliliter on the third day. This was followed by a slow positive drift to an E_h of +0.075 volt and a population of 170×10^7 on the fifth day at which time the culture was discarded. From these results it is apparent that *Esch. coli* can build up a high viable count at an E_h level 200 to 300 mv. more positive than that developed in the ordinary cultures.

SUSPENSIONS OF "RESTING" ESCH. COLI

Forty-eight hour cultures of *Esch. coli*, grown on nutrient agar at 37.5°C., were suspended in saline and washed 3 times by centrifugation. The organisms were then suspended in a mixture of equal parts of M/15 phosphate buffer, pH 7.5, and physiological saline to give a standard turbidity corresponding to a total count of approximately 10×10^9 cells per ml. These suspensions were incubated for two hours at 37.5°C. in shallow sterile dishes in order that the cells might utilize any residual foodstuffs.

Measured volumes of suspensions of these "resting" bacteria, were introduced into the reduction tubes and appropriate amounts of buffer, saline and peptone solutions were added. These suspensions were deaerated for one-half hour before the addition of the standard ferri-ferrocyanide solution. In all tests the concentration of phosphate buffer and of saline was maintained constant. The concentration of reagents employed in a typical test are as follows: 1.0 ml. of a 20 per cent peptone solution in physiological saline, 2.0 ml. of physiological saline solution, 5.0 ml. of M/15 phosphate buffer solution, pH 7.5, 10.0 ml. of the bacterial suspension in the buffer-saline mixture and 2.0 ml. of a 0.1 molar solution of potassium ferricyanide (3 parts)—potassium ferricyanide (1 part). The results of a typical experiment illustrating the influence of different concentrations of peptone, ferricyanide and viable cells on the rate of reduction of ferricyanide are recorded in table 5.

TABLE 5

Influence of the concentration of peptone, oxidant and organisms on the rate of reduction of $K_3Fe(CN)_6$ by a suspension of "resting" Esch. coli, pH 7.5

TOTAL VIABLE COUNT $\times 10^9$	CONCENTRATION OF PEPTONE	INITIAL CONCENTRATION OF $K_3Fe(CN)_6$	MILLIMOLS $\times 10^{-14}$ $K_3Fe(CN)_6$ REDUCED PER CELL PER MINUTE
Influence of peptone concentration			
	per cent	*millimols*	
18.0	10	0.150	3.09
18.0	5.0	0.150	3.95
18.0	3.0	0.150	4.30
18.0	2.0	0.150	4.61
18.0	1.0	0.150	4.33
18.0	0.5	0.150	3.66
18.0	0.1	0.150	1.33
Influence of initial ferricyanide concentration			
18.0	1.0	0.225	6.17
18.0	1.0	0.150	4.33
18.0	1.0	0.075	2.72
18.0	1.0	0.037	1.28
Influence of bacterial concentration			
27.0	1.0	0.150	3.70
18.0	1.0	0.150	4.33
9.0	1.0	0.150	5.50
1.8	1.0	0.150	8.83

The results obtained with suspensions of "resting" *Esch. coli* show that the rate of reduction of ferricyanide increased with increase in the concentration of peptone (other variables being maintained constant) up to 2.0 or 3.0 per cent and then decreased as the concentration of peptone was further increased. The rate of reduction of ferricyanide by well-washed "resting" *Esch. coli* in the absence of peptone or by peptone in the absence of "resting" bacteria was negligible. Duplicate tests agreed within 1.0 per cent of each other and similar tests with suspensions prepared at different times showed the same general behavior and gave results of the same order of magnitude.

The rate of reduction of ferricyanide in a 1.0 per cent peptone solution decreased as the concentration of bacteria was increased. Under the same conditions the rate of reduction of the ferricyanide increased as the original concentration of the ferricyanide was increased. A decrease in temperature of 15°C. reduced the rate of reduction of ferricyanide to approximately one-half of the value observed at 37.5°C.

The hydrogen ion concentration of the suspension exerted a slight influence on the rate of reduction of ferricyanide. Between the pH limits studied, 7.0 and 8.0, the maximum rate was observed at pH 7.5. In a typical experiment the number of millimols $\times 10^{-14}$ of ferricyanide reduced per cell per minute was 5.58, 5.75, and 5.50 at pH 7.0, 7.5, and 8.0, respectively.

In order to study the effect of dead cells on the rate of reduction of ferricyanide a suspension containing 36×10^{8} viable cells per ml. was irradiated with ultra-violet light. These light-killed cells were added to portions of the original suspension and the rate of reduction of ferricyanide determined under conditions as outlined in table 6.

These and similar results indicate that the presence of dead cells has little or no influence on the rate of reduction of ferricyanide.

Various peptone preparations were employed in other tests on the rate of reduction of ferricyanide in identical suspensions of *Esch. coli.* The observed rates of reduction of ferricyanide in millimols $\times 10^{-14}$ per cell per minute at pH 7.5 are as follows:

Bacto-tryptone (Difco) 1.0 per cent solution	5.8
Protease-peptone (Difco) 1.0 per cent solution	5.8
Peptone (Difco or Merck) 1.0 per cent solution	4.8
Neopeptone (Difco) 1.0 per cent solution	4.1
Bacto-protone (Difco) 1.0 per cent solution	2.6

DISCUSSION

The oxidation-reduction potential developed in a 1.0 per cent peptone solution under aerobic conditions is poorly poised in the neighborhood of +0.250 volt (pH 7.0) and on deaeration with a stream of nitrogen gas slowly falls to an equilibrium value near +0.125 volt. During the period of active growth of *Esch. coli* under ordinary aerobic conditions a characteristic drop in potential of 400 to 500 mv. is observed. The maximum reducing potential developed in or near the maximum stationary growth phase of the culture is considerably more negative than that developed in either a deaerated peptone solution or in the filtrate (L 3 Chamberland) of a portion of the culture. Vigorous aeration of the culture results in the temporary establishment of a more positive potential while stirring with nitrogen has little effect on the observed E_h. A positive drift in potential is observed, concurrent with a decrease in the viable count as the age of the culture increases.

TABLE 6

Influence of light-killed cells on the rate of reduction of $K_3Fe(CN)_6$ *in a 1.0 per cent peptone solution, pH 7.5*

VIABLE CELLS ADDED	KILLED CELLS ADDED	RATE OF REDUCTION OF $K_3Fe(CN)_6$ MILLIMOLS × 10^{-14} PER VIABLE CELL PER MINUTE
cc.	*cells*	
10		3.88
10	5	3.88
5		4.00
5	5	3.94

In the stationary cultures the rate of reduction of ferricyanide per cell per minute tends to decrease with increasing age of the cells. The results obtained with suspensions of "resting" coli (tables 5 and 6) suggest that the rate of reduction should increase as the number of viable cells decreases. Some evidence of this is observed in the data presented for the older cultures. The general tendency for the rate of reduction of ferricyanide per cell per minute to decrease with increasing age of the culture may be due to the depletion of readily utilizable foodstuffs (evidence of which has been presented in this communication) to the effect on the cells of the accumulation of waste products, and to physiological differences between cells of different ages.

During the most active period of growth of *Esch. coli* the rate of reduction of ferricyanide ranged from 2.45×10^{-13} to 5.62×10^{-14} millimols per cell per minute. Martin (1932) reported an oxygen consumption of the same order of magnitude (4.0×10^{-13} to 4.0×10^{-14} millimols per cell per minute) during the early stages of growth of *Esch. coli* and likewise noted a tendency for the oxygen consumption per cell to decrease with increasing age of the culture. Walker and Winslow (1932) reported a carbon dioxide production of 0.5×10^{-14} millimol per cell per minute during the period of stable population in aerated peptone cultures of *Esch. coli*, a value of the same order of magnitude as that observed for the reduction of ferricyanide in similar cultures.

In the continuous flow cultures a maximum reduction potential, somewhat greater than that observed in the stationary cultures, is developed and maintained. The viable count also remains at a maximum stationary level, somewhat higher than the maximum developed in the stationary cultures. The rates of reduction of ferricyanide in the continuous flow cultures are, in general, quite constant and greater than those observed in the stationary cultures. In these continuous flow cultures fresh peptone solution is supplied constantly, and rapidly mixes with the culture while an equivalent amount of the culture is continuously removed. We may conclude that the cells in the continuous flow cultures are on the average metabolically similar to those in the latter phases of active growth in ordinary cultures.

The mechanism for the development of the potentials observed in peptone cultures of *Esch. coli* may be postulated as follows: As growth begins an aerobic type of metabolism occurs for a varying time during which the bacteria utilize oxygen and other oxidizing agents in the media. As the concentration of these reagents is decreased and the total metabolic activities of the cells increase, the potential falls and an anaerobic type of metabolism predominates in the culture. The potential reaches a maximum reducing value when the total metabolic requirements of the cells are at a peak value and all available oxidation-reduction systems are employed to meet these demands. In continuous flow cultures this potential is maintained for the above reasons, in stationary cultures a positive shift occurs as the metabolic requirements decrease. The mechanism for the development of reducing conditions as outlined above is in agreement with that postulated by Boyd and Reed (1931) and Hewitt (1933).

The data presented in this communication suggest a possible explanation for the decrease with time in the rate of growth of bacteria and the inability of the bacterial population to increase above a rather definite maximum under a given set of conditions. The total amount of ferricyanide reduced per unit time increases as the concentration of bacteria is increased, other variables being maintained constant. However, this increase is not proportional to the increase in bacterial population, and the amount of ferricyanide reduced *per cell per minute* actually decreases as the concentration of bacteria is increased.

This behavior is also illustrated by the decrease, with increasing age of the culture, in the rate of reduction of ferricyanide per cell during the early phases of growth and to some extent after a maximum population has been established. Approximately 3×10^6 molecules of ferricyanide are reduced per cell per minute in a culture in or near the maximum stationary growth phase while as many as 15×10^6 or more molecules may be reduced per cell per minute in or near the logarithmic growth period. A similar tendency is noted in the reports in the literature on the rate of oxygen consumption, carbon dioxide production, etc., in cultures of *Esch. coli* and other organisms.

The rate of reduction of ferricyanide per cell increases as the concentration of ferricyanide or of peptone is increased. This again is not proportional to the concentration of ferricyanide or of peptone and holds only within certain limits because high concentrations of ferricyanide may exert a toxic action and concentrations of peptone above 3.0 per cent reduce the rate of reduction.

These considerations suggest that the rate of the metabolic activities per cell under a given set of conditions is controlled by the concentration of foodstuffs, oxidants, and cells. The maximum activity per cell is noted when sufficient foodstuffs and oxidant are sorbed and activated (if necessary) in unit time to provide, upon interreaction, for the maximum respiratory and metabolic requirements of the cells.

The change, increasing with age of a culture, in the rate of metabolic activity of the cell may be interpreted on a probability basis. As the number of bacteria increases the concentration gradient of foodstuffs and of oxidants between the cell and its environment decreases, and therefore, the probability of sufficient materials being available per cell per unit time decreases. This decrease may account for the decrease in rate of growth observed in cultures of bacteria in which a relatively high population has been established. An increase in concentration of foodstuffs or oxidant increases, within limits, the probability of sufficient energy and building material being available per cell per unit time.

Other factors (Rahn, 1932) play important rôles in regulating the time-growth relationships in bacterial cultures. The hypothesis presented in this communication does, however, present a relatively neglected aspect of certain of the factors involved in controlling the growth of bacteria.

SUMMARY

Growth, oxidation-reducton potentials, and ferricyanide reduction have been studied in stationary and continuous flow peptone cultures of *Esch. coli*.

A marked fall in potential occurs during the period of rapid growth and a maximum reduction potential is developed in or near the maximum stationary growth period. This potential

and maximum viable count is maintained quite constant in continuous flow cultures.

The concentrations of peptone, organisms and ferricyanide play closely connected rôles in controlling the metabolic activities of the cells, as measured by the rate of reduction of ferricyanide.

Evidence is presented which suggests that the oxidation-reduction potentials observed in bacterial cultures are a resultant of the metabolic activities of the cells.

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OXIDATION-REDUCTION POTENTIALS AND FERRICYANIDE REDUCING ACTIVITIES IN GLUCOSE-PEPTONE CULTURES AND SUSPENSIONS OF ESCHERICHIA COLI

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The oxidation-reduction potentials developed in stationary and continuous flow peptone cultures of *Esch. coli* and the rates of reduction of ferricyanide by these cultures and by suspensions of "resting" *Esch. coli* in the presence of peptone have been reported in the first paper of this series (Clifton, Cleary, and Beard, 1934). The studies to be reported here deal with the influence of glucose on the oxidation-reduction potentials and ferricyanide reducing activities in glucose-peptone cultures of *Esch. coli* and of glucose, lactose, and maltose on the ferricyanide reducing activities in suspensions of "resting" *Esch. coli* under the same conditions described in the first paper.

EXPERIMENTAL RESULTS

Stationary and continuous flow glucose-peptone cultures

Under aerobic conditions at 37.5°C. the oxidation-reduction potential of a 1.0 per cent Difco peptone, 0.25 per cent glucose (0.5 per cent NaCl, M/50 phosphates, pH 7.4) solution was poorly poised in the neighborhood of +0.225 volt. Characteristic time-potential, time-population and time-pH relationships are recorded in figure 1 for stationary (ordinary aerobic) and continuous flow (250 ml. daily) cultures of *Esch. coli* (laboratory strain K-12) in this phosphate-glucose-peptone medium.

A corresponding behavior was noted when the concentration of glucose was increased to 0.5 per cent. The positive drift in potential was somewhat more rapid than that observed in the 0.25 per cent glucose cultures, shifting from a maximum reduction potential of −0.320 volt at the end of the fifth hour to a level of −0.033 volt at the end of the twenty-fourth hour.

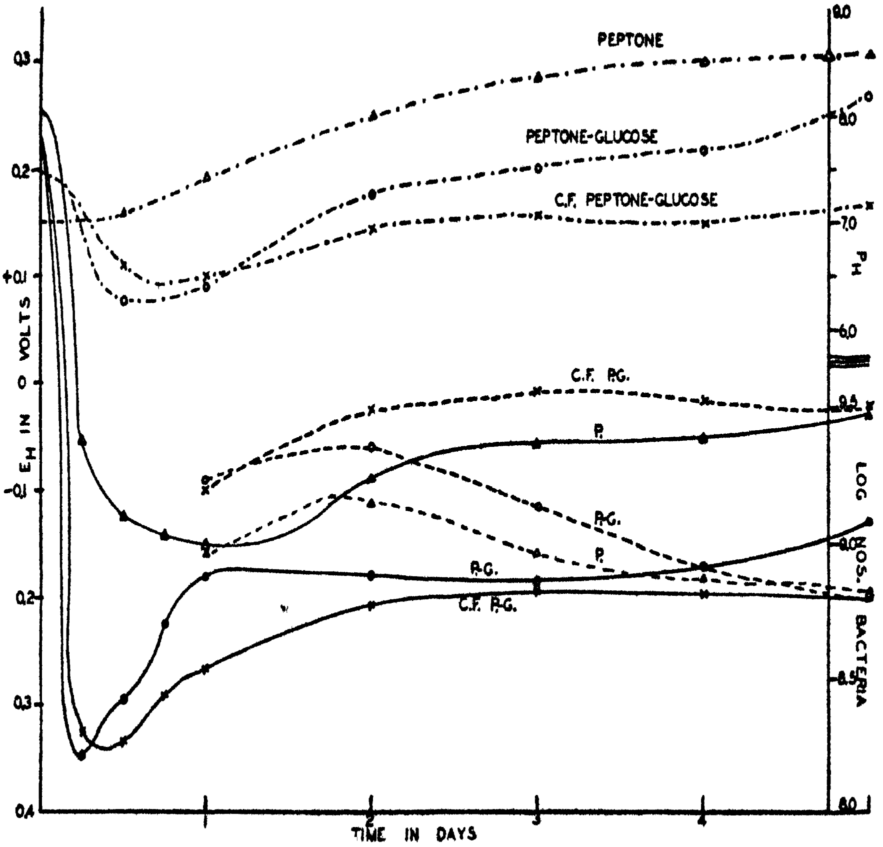

FIG. 1. TIME-POTENTIAL (—), TIME-POPULATION (— —) AND TIME-pH (-.-.-) RELATIONSHIPS IN STATIONARY (o) AND CONTINUOUS FLOW (x) (250 cc. DAILY) 1.0 PER CENT PEPTONE, 0.25 PER CENT GLUCOSE CULTURES OF *Esch. coli* AND IN A STATIONARY PEPTONE CULTURE (Δ)

During this period of time the pH decreased to 4.5 and was maintained at this low level, even in certain experiments in which the phosphate buffer content was increased to M/20 and sterile N/2 NaOH added daily to neutralize the acids formed in the culture.

It is apparent from these results that either the sugar was completely utilized in the 0.25 per cent glucose cultures and the pH then shifted to a more alkaline value or the rate of fermentation of glucose decreased as the age of the culture increased and basic products of peptone degradation neutralized the non-utilizable acids formed during fermentation. In order to ascertain the extent of sugar utilization quantitative glucose determinations were carried out after twenty-four hours growth of *Esch. coli* in 0.25 and 0.5 per cent glucose, 1.0 per cent peptone, M/50 phosphate media. The concentration of glucose decreased from 0.25 to 0.07 per cent in the first culture and from 0.50 to 0.21 per cent in the richer medium.

TABLE 1

Rates of reduction of $K_3Fe(CN)_6$ by 1.0 per cent peptone, 0.25 per cent glucose, M/50 phosphate cultures of Esch. coli

AGE OF CULTURE	STATIONARY CULTURE		CONTINUOUS FLOW CULTURE	
	Plate count per ml. × 10^7	Millimols $K_3Fe(CN)_6$ reduced per cell per minute	Plate count per ml. × 10^7	Millimols $K_3Fe(CN)_6$ reduced per cell per minute
hours				
24	174	5 74 × 10^{-14}	157	4.24 × 10^{-14}
48	227	1 52 × 10^{-14}	315	2.84 × 10^{-14}
72	137	2.21 × 10^{-14}	370	5 78 × 10^{-14}
96	83	1.79 × 10^{-14}		
120	63	1.50 × 10^{-14}		

Ferricyanide reduction tests were carried out with these cultures by the method described in the first paper of this series (Clifton, Cleary and Beard, 1934). Typical results are recorded in table 1.

These and similar results on the rate of reduction of ferricyanide per cell by stationary glucose-peptone cultures of *Esch. coli* show the same general tendency to decrease as the age of the cultures increases which was observed in plain peptone cultures. The rate tends to remain more constant in the continuous flow cultures but the results of these experiments are only suggestive as they do not extend over a sufficient period of time. Difficulties were encountered in maintaining a constant volume in the continuous flow cultures as a result of blockage of the automatic siphon by evolved gases and the clogging of the capillaries by phosphate-peptone precipitates.

SUSPENSIONS OF "RESTING" ESCH. COLI

Suspensions of "resting" bacteria (in M/30 phosphates, 0.45 per cent NaCl, pH 7.4) were prepared and their reducing activities at 37.5°C. determined in the same manner as described in the first paper of this series (Clifton, Cleary and Beard, 1934). Glucose, maltose, lactose and peptone were employed as substrates and potassium ferricyanide, o-cresol indophenol, methylene blue, indigotetrasulfonate or indigodisulfonate as the oxidant.

The results obtained with a typical suspension, illustrating the influence of different concentrations of glucose, ferricyanide and viable cells on the rate of reduction of ferricyanide are recorded in table 2.

These results show that the rate of reduction of ferricyanide per cell in suspensions of "resting" *Esch. coli* increased to a maximum in a 2.0 per cent glucose solution. The concentration of glucose does not appear to exert as great an influence on the rate of reduction of ferricyanide as does the concentration of peptone in similar tests. Duplicate tests agreed within 1.0 per cent of each other and similar tests with suspensions prepared at different times showed the same general behavior and gave results of the same order of magnitude. The rate of reduction of ferricyanide by well-washed "resting" bacteria in the absence of glucose or by glucose in the absence of "resting" bacteria was negligible.

The rate of reduction of ferricyanide in a 1.0 per cent glucose solution decreased as the concentration of bacteria was increased. Under the same conditions the rate of reduction of ferricyanide increased as the original concentration of the ferricyanide was increased.

A similar behavior was observed when maltose or lactose was substituted for glucose. Typical results obtained with these sugars with and without peptone are given in table 3.

TABLE 2

Influence of the concentration of glucose, oxidant, and organisms on the rate of reduction of $K_3Fe(CN)_6$ by a suspension of "resting" Esch. coli, pH 7.5

TOTAL VIABLE COUNT $\times 10^9$	CONCENTRATION OF GLUCOSE	CONCENTRATION OF $K_3Fe(CN)_6$	MILLIMOLS $\times 10^{-14}$ $K_3Fe(CN)_6$ REDUCED PER CELL PER MINUTE
Influence of glucose concentration			
	per cent	*millimols*	
9.0	4.0	0.150	7.14
9.0	2.0	0.150	8.88
9.0	1.0	0.150	8.00
9.0	0.5	0.150	7.78
9.0	0.1	0.150	7.33
Influence of ferricyanide concentration			
9.0	1.0	0.225	9.44
9.0	1.0	0.150	8.00
9.0	1.0	0.075	3.66
Influence of bacterial concentration			
13.5	1.0	0.150	7.79
9.0	1.0	0.150	8.00
4.5	1.0	0.150	8.44

TABLE 3

Influence of peptone, glucose, maltose and lactose on the rate of reduction of $K_3Fe(CN)_6$ by a suspension of "resting" Esch. coli, pH 7.5

SUBSTRATE (0.5 PER CENT CONCENTRATION OF EACH)	TOTAL VIABLE COUNT	MILLIMOLS $\times 10^{-14}$ $K_3Fe(CN)_6$ REDUCED PER CELL PER MINUTE
Peptone	20×10^9	6.20
Glucose	20×10^9	6.40
Peptone + glucose	20×10^9	10.00
Maltose	20×10^9	7.52
Peptone + maltose	20×10^9	7.65
Lactose	20×10^9	2.35
Peptone + lactose	20×10^9	6.90

Under the conditions of the experiments reported in table 3 the rate of reduction of ferricyanide was greatest in the presence of maltose and decreased in the order given for the following substances: glucose, peptone, and lactose. The addition of peptone to glucose, in various concentrations of each substrate, increased the rate of reduction. The rate of reduction of ferricyanide was not markedly increased above the value observed with peptone alone when maltose or lactose was added to the suspension of "resting" bacteria in the presence of peptone. The marked increase in the rate of reduction per cell observed when peptone was added to the glucose solution is of particular interest as no marked increase was noted when peptone was added to maltose, which appears to be the most readily utilized sugar of those tested.

Ferricyanide was not discernibly toxic in the concentration employed during the course of these experiments which were ordinarily completed within sixty to ninety minutes nor was any appreciable multiplication observed.

In similar experiments potassium ferricyanide was replaced as the oxidant by certain of the oxidation-reduction indicators developed by Clark, the change in ratio of oxidant to reductant being followed potentiometrically and checked visually at various intervals of time. Molar solutions, 0.01 (or saturated aqueous solutions of the less soluble dyes) of o-cresol indophenol, methylene blue, and indigo tetra- or disulphonate were employed.

o-Cresol indophenol was apparently reduced rather rapidly by peptone, maltose or glucose activated by "resting" coli. Due to the low solubility of this dye and its instability under the conditions of these tests the results obtained were rather erratic.

Methylene blue was rapidly reduced under the conditions of these experiments by peptone, maltose and glucose in the order named in the presence of *Esch. coli.* The reduction of methylene blue by colon bacilli in lactose was negligible. Neither the substrates nor "resting" bacteria reduced methylene blue at an appreciable rate. Typical results of the methylene blue reduction tests are graphically recorded in fig. 2.

No marked reduction of the indigo sulfonates was observed in peptone, glucose, maltose, or lactose and the potential appeared to be stabilized near the upper potential levels of these systems.

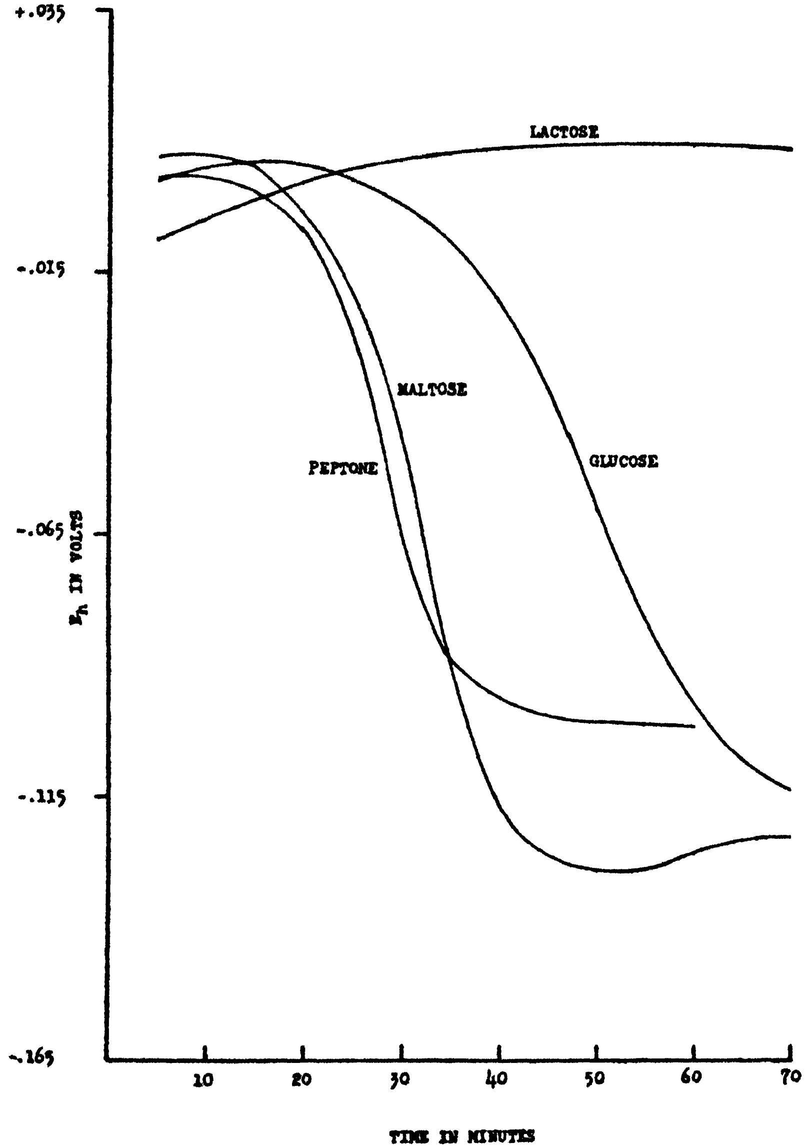

FIG. 2. TIME-POTENTIAL CURVES REPRESENTING THE ADDITION OF METHYLENE BLUE (FINAL CONCENTRATION 0.001 MOLAR) TO DEAERATED SUSPENSIONS OF *Esch. coli* IN BUFFERED, 1.0 PER CENT SOLUTIONS OF PEPTONE, MALTOSE, GLUCOSE, AND LACTOSE, pH 7.5

DISCUSSION

During the period of active growth of *Esch. coli* under aerobic conditions in a 0.25 per cent glucose, 1.0 per cent peptone medium a rapid drop in potential was observed to the relatively low level of −0.340 to −0.350 volt at pH 6.5. As the growth rate decreased a slow positive drift in potential occurred and a level of −0.180 volt at pH 6.4 was reached within twenty-four to thirty hours. The pH shifted to 7.3 within the next twenty-four hours and then slowly increased to 8.2 by the fifth day at which time the culture was discarded.

Similar time-potential and time-pH relationships were noted in the continuous flow cultures (250 ml. daily flow) with the exception that the pH appeared to be stabilized between 7.0 and 7.2 and the potential at −0.200 volt by the third day. In cultures containing higher concentrations of glucose the pH shifted from 7.4 to 4.6 or less within twenty-four hours and tended to remain at this level.

Glucose and pH determinations in the 0.25 per cent glucose, 1.0 per cent peptone cultures indicate that the concentration of glucose was markedly decreased (0.25 to 0.07 per cent) during the first twenty-four hours growth of *Esch. coli* and suggest that basic products of peptone degradation neutralized the non-utilizable acids formed during the fermentation of glucose. The time-potential relationships also suggest that glucose metabolism predominates during the early phases of growth and that the potential then shifts to a level near that observed in a plain peptone culture as the concentration of glucose further decreases with increasing age of the culture.

In a continuous flow (250 ml. daily, 0.25 per cent glucose, 1.0 per cent peptone solution) culture of *Esch. coli* the same considerations apply. The potential was stabilized within two days at a somewhat lower level than in the stationary cultures and the stabilization of pH at 7.0 to 7.2 suggests that the non-utilizable acids produced by fermentation were neutralized by basic substances liberated during the metabolism of peptone. The stabilization of the oxidation-reduction potential in the neighborhood of −0.200 volt indicates that a higher reducing intensity was developed in the continuous flow glucose-peptone cultures than in similar sugar free cultures. This suggests that an equilibrium is established between the rate of glucose and peptone metabolism, concomitantly with the establishment of a maximum stable population.

In the stationary cultures the rate of reduction of ferricyanide per cell tends to decrease with increasing age of the cells. In the continuous flow cultures the rate of reduction tends to remain constant. The same considerations apply to these cultures as outlined in the first paper of this series.

Of the sugars tested maltose appears to be most readily oxidized in suspensions of "resting" *Esch. coli* when ferricyanide or methylene blue is employed as the oxidant. Glucose is oxidized somewhat less rapidly than maltose under the same conditions. The rate of ferricyanide reduction by lactose plus "resting" coli is less than one-half that observed with maltose or glucose while the rate of reduction of methylene blue by lactose under the same conditions is negligible.

SUMMARY

Growth, oxidation-reduction potentials, and ferricyanide reduction have been studied in stationary and continuous flow glucose-peptone cultures of *Esch. coli.*

A more marked fall in potential occurs during the period of rapid growth and a higher reducing intensity is established in the glucose-peptone cultures than is observed in plain peptone cultures. A higher maximum viable population is also developed in the presence of glucose and this maximum population is maintained under favorable conditions in continuous flow cultures.

The concentrations and nature of the substrates and oxidants and the concentration of organisms play closely connected rôles in controlling the metabolic activities of the cells, as measured by the rate of reduction of ferricyanide.

Maltose, glucose and lactose are readily oxidized by potassium ferricyanide in suspensions of "resting" *Esch. coli.* These limited studies suggest that the ability of coli to utilize these sugars decreases as the E_h of the oxidant decreases.

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STUDIES ON CERTAIN PHYSIOLOGICAL CHARACTERS OF PHYTOMONAS TUMEFACIENS, PHYTOMONAS RHIZOGENES, AND BACILLUS RADIOBACTER

PART I[1]

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INTRODUCTION

A comparison has been made of certain physiological characters of *Phytomonas tumefaciens* (Smith and Town.) Bergey, *et al.*, and *Phytomonas rhizogenes* Riker *et al.*, the cause of crown gall and hairy root respectively, and of *Bacillus radiobacter*, Beij. and Van Deld., a soil saprophyte similar in many physiological characters to *P. tumefaciens*. Since it appeared that the influences of these organisms in plants were doubtless physiological, depending in part upon the utilization of food materials within the tissue, it seemed desirable to determine how they would act on nutrients of known composition.

These studies centered about the following: (1) the utilization of various nitrogen-containing compounds; (2) the use of various carbon sources with certain sources of nitrogen; (3) nitrate reduction in the presence of certain sugars; and (4) the oxidation-reduction potentials induced in certain media.

The early work with these bacteria was primarily concerned with their identification. Descriptions of the crown-gall organism may be found in a number of papers including those by Smith and Townsend (1907), Smith, Brown and Townsend (1911), Patel (1929), Muncie and Suit (1930), Riker *et al.*, (1930), and Wright *et al.*, (1930); *B. radiobacter* by Beijerinck and Van Delden (1902), Löhnis (1905), Löhnis and Hansen (1921), Israilsky (1929), Muncie and Suit (1930), Riker *et al.*, (1930); and the hairy-root organism by Muncie and Suit (1930), Riker *et al.* (1930), and Wright, *et al.* (1930). The descriptions of the three bacteria in these and other papers show certain discrepancies omitted here because of the space required for their discussion. It appears that the most valid descriptions are those made with single-cell cultures.

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CULTURES OF BACTERIA EMPLOYED

The sources of the cultures employed were diverse. The majority of the strains employed were progenies of single cells. The nine crown-gall cultures used were from raspberry (see T–5, etc., Wright *et al.* 1930). The five hairy-root cultures were obtained: two from a mixed infection on walnut (see T–37–1a and T–37–2b, Riker *et al.*, 1930) and three from apple (see C–1, D–20, and T–38–1a, Wright *et al.*, 1930). The radiobacter cultures were obtained: six from the United States Department of Agriculture and four locally (see R–1, etc., Riker *et al.*, 1930).

The identity of all cultures was determined by differential cultural tests and by reactions on host plants repeated from time to time during the work as described by Riker *et al.* (1930).

UTILIZATION OF NITROGEN

Since no comparisons were found of the utilization by these organisms of nitrogen-containing compounds, representative sources of nitrogen were prepared in liquid media. Their utilization was determined either by macroscopic growth or, in some cases, by stained preparations.

Determinations were made in the following medium: magnesium sulphate ($MgSO_4 \cdot 7H_2O$), 0.2 gram, sodium chloride ($NaCl$), 0.2 gram, calcium chloride ($CaCl_2$), 0.1 gram, dibasic potassium phosphate (K_2HPO_4), 0.2 gram, a nitrogen source (see table 1), 5 grams, and a carbon source, 5.0 grams in 1000 cc. of water. The nitrogen source, and the salt-carbohydrate were prepared separately in double strength, neutralized, sterilized, mixed and tubed aseptically.

The potato extract was prepared by thoroughly cooking two hundred grams of potato, sliced into sections about one-fourth inch in thickness, in one-half liter of distilled water. This extract, filtered twice through absorbent cotton, neutralized, made up to one-half liter and autoclaved, was added to a sterile double-strength solution of glucose and pipetted into sterile test tubes.

The yeast-water extract, used at the rate of 100 cc. of clear supernatant liquid in each liter of medium, was prepared by autoclaving 100 grams of starch-free yeast in a liter of distilled water.

The amino acids, tyrosine, alanine, glutamic acid and cystine, were used singly and in combination at the rate of one and one-fourth grams each per liter, with and without traces of ferrous sulphate or ferric chloride. Ammonium citrate and ferric ammonium citrate were used in agar shake and agar stab cultures, and also in the liquid media. In order to remove any soluble nitrogen or carbon compounds from the agar it was washed in repeated changes of distilled water so that when the nitrogen source was withheld from the medium, no growth occurred with any of the bacteria. The cultures were seeded from slant cultures forty-eight to seventy-two hours old and incubated at 28°C. for three weeks.

INORGANIC NITROGEN

It may be noted in table 1 that *P. rhizogenes* cultures differed from those of crown-gall and *B. radiobacter* because they produced little or no growth with the inorganic nitrogen compounds, while the other two organisms grew well except with potassium nitrite. With ammonium citrate and ferric ammonium citrate, both in liquid and agar shake cultures, *P. tumefaciens* and *B. radiobacter* grew very well with a browning of the medium in the upper layer. *P. rhizogenes* induced an acid reaction with ammonium citrate, and ferric ammonium citrate which differentiated it from the other two. *B. radiobacter* was differentiated by the alkaline reaction it induced with potassium nitrate.

TABLE 1

*Summary of studies on growth and hydrogen-ion concentration produced by Phytomonas tumefaciens, Phytomonas rhizogenes, and Bacillus radiobacter in media containing various sources of nitrogen and carbon**

SOURCE OF NITROGEN	SOURCE OF CARBON	GROWTH† OF *P. rhizogenes* (5 STRAINS)	HYDROGEN-ION CONCENTRATION WITH			
			Control	*P. tumefaciens* (9 strains)	*P. rhizogenes* (5 strains)	*B. radiobacter* (10 strains)
None	Glucose	0	6.8	6.8	6.8	6.8
Inorganic						
Ammonium sulphate	Glucose	+‡	6.4	5.8	5.8	5.8
Ammonium sulphate	Sucrose	–	6.4	5.2		5.2
Ammonium sulphate	Mannitol	–	6.4	4.6		4.8
Ammonium citrate	Glucose	+	6.6	9.0	5.8	9.0
Ferric ammonium citrate	Glucose	+	7.0	7.2	4.7	7.2
Ammonium nitrate	Glucose	0	6.0	5.8	6.0	5.8
Ammonium nitrate	Glycerine	+‡	6.2	6.2	6.0	8.2
Potassium nitrite	Glucose	0	7.4	6.8	7.4	6.8
Potassium nitrate	None	0	6.8	6.8	6.8	6.8
Potassium nitrate	Glucose	0	7.2	7.2	7.2	9.2
Organic						
Methylamine	Glucose	0	7.2	6.0	7.2	6.0
Acetamide	Glucose	+‡	6.4	6.2	6.2	6.4
Oxamide	Glucose	++	6.2	5.0	5.0	5.2
Succinimide	Glucose	+	6.6	7.0	6.6	7.2
Dicyandiamide	Glucose	++	7.0	6.6	6.4	6.6
Asparagine	Glucose	++	6.6	8.4	5.8	8.4
Urea	Glucose	+‡	7.6	9.2	8.2	9.2
Urea	Sucrose	+‡	7.8	9.2	8.2	9.2
Urea	Mannitol	++	8.0	9.0	8.0	9.2
Uric acid	Glucose	+	6.4	6.6	5.4	6.8
Amino acids						
Glycine	Glucose	+	6.4	6.8	6.4	8.0
dl-Alanine	Glucose	+‡	6.6	7.8	6.4	8.1
dl-Alanine	Mannitol	+	7.0	6.6	6.6	6.6
l-Tyrosine	Glucose	+	6.8	4.8	4.8	4.8
l-Tyrosine	Mannitol	+++	6.8	5.4	6.0	5.8
l-Cystine	Glucose	+‡	6.8	4.8	5.2	4.8
l-Aspartic acid	Glucose	++	7.2	9.0	4.8	9.0
l-Aspartic acid	Mannitol	+	7.2	8.8	5.2	8.8
d-Glutamic acid	d-Glutamic acid	–	7.2	8.8		9.2
d-Glutamic acid	Glucose	+++	7.6	7.8	4.6	7.8
d-Glutamic acid	Mannitol	–	7.0	8.0		8.0

TABLE 1—*Concluded*

SOURCE OF NITROGEN	SOURCE OF CARBON	GROWTH† OF *P. rhizogenes* (5 STRAINS)	HYDROGEN-ION CONCENTRATION WITH			
			Control	*P. tumefaciens* (9 strains)§	*P. rhizogenes* (5 strains)	*B. radiobacter* (10 strains)§
Amino acids—*Concluded*						
d-Glutamic acid	Sucrose	–	7.0	7.2		7.2
l-Leucine	Glucose	–	6.8	5.4		5.2
Mixture	Glucose	+‡	7.2	8.0	7.2	8.0
Mixture + $FeSO_4$	Glucose	+‡	7.0	7.2	6.6	7.2
Mixture + $FeCl_3$	Glucose	+‡	7.0	8.0	7.0	8.0
Miscellaneous						
Peptone	Peptone	++++	7.4	8.2	8.2	8.2
Peptone	Arabinose	+++	6.6	6.3	5.4	6.2
Peptone	Xylose	++++	6.4	6.3	4.4	6.4
Peptone	Glucose	++++	6.8	6.6	4.7	6.6
Peptone	Mannose	+++	6.6	6.6	4.6	6.6
Peptone	Sucrose	++++	6.6	6.6	6.2	6.4
Peptone	Lactose	+++	6.4	6.6	5.2	6.6
Peptone	Raffinose	+++	6.8	6.8	6.8	6.8
Peptone	Salicin	++	6.2	6.1	4.6	6.2
Peptone	Erythritol	+++	6.8	7.4	4.6	7.4
Peptone	Mannitol	+++	6.4	6.4	6.2	6.4
Yeast-water extract	Glucose	++++	6.6	6.5	4.6	6.6
Potato extract	Potato extract	++++	6.6	9.2	9.2	9.2
Potato extract	Glucose	+++	6.8	7.4	4.8	7.6

* The composition of the medium employed is given in the text. The final readings were made after three weeks incubation at 28°C.

† Growth is indicated as follows: 0 = none, + = trace, ++ = slight, +++ = moderate, ++++ = abundant, – = no determinations made.

§ *P. tumefaciens* and *B. radiobacter* each produced moderate to abundant growth in all cases except in the potassium nitrite medium and controls lacking either a carbon or a nitrogen source.

‡ Microscopic examination also made and compared with controls.

AMIDES

The hairy-root cultures gave limited development with nitrogen of simple organic compounds (table 1). No growth was secured with methylamine. Compounds such as acetamide, oxamide, succinimide, dicyandiamide, asparagine, urea, and uric acid were utilized readily by *P. tumefaciens* and *B. radiobacter* while *P. rhizogenes* produced only a trace to a slight amount of growth. With urea, all three organisms induced an alkaline reaction, whereas with the other compounds there were some differences.

AMINO ACIDS

With the various amino acids, and with glucose or mannitol as a carbon source, the crown-gall and *B. radiobacter* cultures produced good growth, but *P. rhizogenes* produced only a trace, except with aspartic acid, glutamic acid, and tyrosine. Both carbon and nitrogen for *P. tumefaciens* and *B. radiobacter* were provided by glutamic acid.

An alkaline reaction was induced by *B. radiobacter* and *P. tumefaciens* when glycine, alanine, aspartic acid, or glutamic acid were used with glucose, while hairy-root cultures induced an acid reaction. All three organisms induced an acid reaction with tyrosine and cystine. The first two mentioned organisms induced an acid condition with leucine.

Mixtures of amino acids, tyrosine, alanine, glutamic acid, and cystine, with and without the addition of iron salts, were tested. *P. rhizogenes* produced only a trace of growth, while *B. radiobacter* and *P. tumefaciens* produced good growth.

MISCELLANEOUS NITROGENOUS SUBSTANCES

Difco peptone, yeast infusion and potato extract, as seen in table 1, appear to be the most suitable sources of nitrogen for the growth of all three organisms. The hairy-root organism grew better with one or another of these complex substances than with the other nitrogen-containing compounds.

Difco peptone was utilized as the sole source of nitrogen and carbon by all three organisms. Where peptone was used alone, all three organisms induced an alkaline reaction due to the formation of ammonia, as determined by testing with Nessler's solution.

The hairy-root cultures induced a strong acid reaction in media containing peptone and a carbohydrate, except with raffinose, sucrose, and mannitol. It is probable that these carbohydrates were less readily attacked than the peptone, with the formation of ammonia which neutralized the acids formed.

Yeast-water extract when used with glucose gave results very similar to those secured from peptone with glucose. A decided acid reaction, pH 4.6, was induced by *P. rhizogenes,* while the reaction remained practically unchanged with the others. Crown-gall cultures produced a fairly heavy membrane, but not nearly as heavy as *B. radiobacter* cultures.

With the potato extract alone, i.e., with no further salts or carbohydrates, the three groups of organisms each produced good growth with an alkaline reaction. When potato extract was used with glucose, the hairy-root cultures induced a strongly acid reaction, pH 4.8, while the other two types of organisms induced a slightly alkaline reaction, pH 7.4 and 7.6.

The type of growth of the three organisms in the various media was in many instances quite different. The crown-gall organism produced a heavy membrane in most cases, accompanied by a veil, with an otherwise clear liquid. *P. rhizogenes* usually produced a rather uniform turbidity in the broth, with slight if any membrane or veil. *B. radiobacter* produced a heavy membrane in most cases with an accompanying uniform turbidity of the medium, and a moderate-to-abundant sediment.

P. rhizogenes seems to lack the ability of *P. tumefaciens* and *B. radiobacter* to utilize the simpler nitrogenous compounds. The hairy-root organism thrived best with the more complex sources of nitrogen. With the simpler nitrogen-containing compounds, growth was either absent or very meager and seldom increased after the third or fourth day, while the other two organisms grew well in almost every case. This might result in part from the inability of the organism to use the ingredients of the medium, but other factors may be influential.

NITRATE NITROGEN WITH A REDUCING AGENT

Since *P. rhizogenes* failed to produce growth in a medium containing 0.5 per cent potassium nitrate, it was considered that this might result from an unfavorable oxidation-reduction potential. By the addition of sterile sand, sterile divided filter paper, or sterile agar to tubes of liquid culture medium, which was ordinarily too highly oxidized to permit growth of root-nodule bacteria, Allyn and Baldwin (1930) induced growth which, they considered, was due to trapping of small volumes of the medium in which the organisms were able to make the necessary adjustments. Similar trials, with sterile washed sand and sterile agar, were made with *P. rhizogenes* in mannitol and glucose media containing potassium nitrate at the rate of 0.5, 1.0, and 5.0 grams per liter. The carbohydrate was autoclaved separately from the other constituents.

The inocula were 0.2 cc. per 10 cc. of medium of suspensions from two-day-old cultures prepared in sterile distilled water and standardized to a turbidity which would conceal a two millimeter loop, made from No. 26 B. and S. gauge wire, at twenty millimeters depth. After seeding, sterile washed sand or sterile agar was added under aseptic conditions to give a column of ten millimeters in the bottom of the tubes. With the use of the higher concentration of potassium nitrate no growth was initiated, while growth occurred with the two lower concentrations.

Reducing agents were used by Allyn and Baldwin (1930) to facilitate the growth of *Rhizobium* cultures in media which would otherwise be too highly oxidized. Similar trials, in which fresh potato blocks were used as the reducing agent in Petri dishes containing the glycerophosphate medium employed by Riker *et al.* (1930), were made. The growth of *P. rhizogenes* was favored. There is, however, a possibility that diffusion of the nutrients from the potato block into the surrounding medium favored growth, rather than merely the lower oxidation-reduction potential.

Thioglycollic acid was also used as a reducing agent with the mannitol and glucose nitrate media. The potassium nitrate was used in varying amounts, namely, 0.5, 1.0, and 5 grams per liter, while the thioglycollic acid was used at the rate of 0.025 to 0.15 gram per liter. The sugar and salts were prepared separately, autoclaved, mixed aseptically and pipetted in 8-cc. portions to sterile test tubes. One cubic centimeter of the bacterial suspension as earlier described and 1 cc. of the diluted and neutralized thioglycollic acid were added to each tube. The thioglycollic acid was found to be sterile, and was prepared by dilution in sterile distilled water and neutralized with sterile N/14 NaOH. Brom-thymol blue was added to check the hydrogen-ion concentration. The use of the nonnitrogenous reducing agent, thioglycollic acid, gave results similar to those with sand. Growth was initiated with the lower concentrations of potassium nitrate, and with all the concentrations of thioglycollic acid used, but did not increase after the third day. The total growth was meager.

No attempts have thus far been made to stimulate growth of *P. rhizogenes*, in the medium with nitrate as the source of nitrogen, by the addition of small amounts of what might be growth promoting substances as accomplished by Reader (1928), Farries and Bell (1930), and others working with various microörganisms.

UTILIZATION OF CARBON SOURCES

The availability of various carbon sources was examined because of certain earlier mentioned discrepancies in previous work. In that work, sources of nitrogen such as peptone were commonly used which might serve also as sources of carbon. In the present studies potassium nitrate was employed, which was readily available for *P. tumefaciens* and *B. radiobacter*. Because it was not used by *P. rhizogenes*, results from this organism were negative and are omitted.

The composition and preparation of the medium, inoculation, and incubation were the same as described under the heading "Utilization of Nitrogen." Carbohydrates, glucosides, alcohols, and organic acids or their salts were employed as sources of carbon, and the results obtained are given in table 2.

SUGARS

The saccharides arabinose, glucose, lactose, sucrose, raffinose, and dextrin were utilized to about the same extent by *P. tumefaciens* and *B. radiobacter*. *P. tumefaciens*, however, was slower in starting growth where lactose was used. *P. tumefaciens* induced no decided change in the hydrogen-ion concentration, but *B. radiobacter* induced an alkaline reaction (pH 7.6 to pH 9.4), probably due to the reduction of the nitrate and corresponding

TABLE 2

*Summary of studies on growth and hydrogen-ion concentration produced by Phytomonas tumefaciens and Bacillus radiobacter in media with various sources of carbon, containing potassium nitrate as a nitrogen source**

SOURCE OF CARBON	GROWTH† OF		HYDROGEN-ION CONCENTRATION WITH		
	P. tumefaciens (9 strains)	*B. radiobacter* (10 strains)	Control	*P. tumefaciens* (9 strains)	*B. radiobacter* (10 strains)
Arabinose	++++	++++	6.8	7.2	9.2
Glucose	++++	++++	7.2	7.2	9.4
Lactose	+++	+++	7.0	7.0	8.0‡
Sucrose	++++	++++	7.0	7.0	7.6‡
Raffinose	++++	++++	7.0	7.2	9.4
Dextrin	++++	++++	7.2	7.4	9.2
Salicin	++++	++++	7.0	6.8	7.6‡
Methyl alcohol	+§	+§	7.2	7.2	7.4
Ethyl alcohol	+++	+++	7.2	4.8	9.2
Propyl alcohol	+++	+++	7.2	5.6	5.4
Butyl alcohol	+§	+§	7.2	7.2	7.2
Glycerol	++++	++++	6.8	7.0	9.2
Mannitol	++++	++++	7.0	7.0	9.2
Adonitol	++	++++	7.4	7.4	8.8
Quercit	+§	++**	7.2	7.2	7 2
Dulcitol	+++	++++	7.0	7.0	7.4‡
Lactositol	+++	++++	7.2	7.2	7.6‡
Arabitol	++++	++++	7.2	7.2	9.2
Sodium formate	+§	+§	7.2	8.4††	8.5††
Sodium acetate	+++	+++	7.0	8.4‡	9.0
Sodium propionate	++++	++++	7.0	9.0	9.0
Butyric acid	++§	++§	6.8	6.8	7.2
Lactic acid	++++	++++	6.8	8.6	9.2
Glycollic acid	++§	++§	6.8	7.8††	7.4††
Calcium gluconate	++++	++++	7.0	7.2	8.6
Stearic acid	++§	++§	7.4	7.4	7.4
Sodium oxalate	0§	0§	7.2	7.2	7.2
Sodium malonate	++§	++§	7.0	7.2	7.2
Sodium succinate	++++	++++	6.8	9.0	9.0
Malic acid	++++	++++	7.0	9.0	9.2
Tartaric acid	++§	++§	7.0	7.2	7.2
Sodium citrate	++++	++++	6.8	9.4	9.6
None	0§	0§	6.8	6.8	6.8

* The composition of the media employed is given in the text. The final readings were made after three weeks incubation at 28°C.

† Growth is indicated as follows: 0 = none, + = trace, ++ = slight, +++ = moderate, ++++ = abundant.

‡ Reaction decidedly alkaline after six weeks.

§ Microscopic examination made.

** Slight brownish-black color.

†† Tested at six weeks.

accumulations of basic ions. With the glucoside, salicin, similar results were induced, with the exception that at three weeks a reaction of pH 7.6 was induced by *B. radiobacter* and one of pH 9.0 at nine weeks.

ALCOHOLS

When ethyl or propyl alcohol was used, equal growth was produced by *P. tumefaciens* and *B. radiobacter*. The crown-gall organism induced an acid reaction, but *B. radiobacter* induced an alkaline reaction with ethyl alcohol and an acid reaction with propyl alcohol. Nitrates disappeared with many of the *B. radiobacter* cultures after nine weeks. With methyl or butyl alcohol no macroscopic evidence of growth was visible, but stained preparations indicated that the organisms were able to start development, but were unable to continue.

With the poly-hydroxy alcohols, mannitol, glycerol, and arabitol, very good growth was produced. The reaction again was decidedly alkaline with *B. radiobacter* but remained unchanged with *P. tumefaciens*. When adonitol, lactositol, and dulcitol were used, considerably more growth was produced by *B. radiobacter* than by *P. tumefaciens*, but not as alkaline a reaction as with the other carbon sources. With quercit, a slight brownish to black color was produced by *B. radiobacter* with slight growth. The crown-gall organism produced only a trace of growth and no discoloration of the medium or change in reaction.

SALTS OF ORGANIC ACIDS

When sodium formate, butyric acid, glycollic acid, stearic acid, sodium malonate, or tartaric acid were used in place of the carbohydrate, neither *P. tumefaciens* nor *B. radiobacter* produced more than slight growth. Only with the use of sodium formate and glycollic acid was there any great change in hydrogen-ion concentration, where growth was slight, and this only after six weeks incubation.

With sodium acetate only moderate growth took place with either of the organisms, but abundant growth appeared with sodium propionate, lactic acid, calcium gluconate, sodium succinate, malic acid, and sodium citrate. The medium became decidedly alkaline in reaction. Where sodium oxalate was used no growth was produced by either organism.

It may be noted that a wide range of carbonaceous materials was utilized by *P. tumefaciens* and *B. radiobacter* (table 2). Both types of microörganisms utilized the compounds to about the same extent. They were, however, differentiated on the basis of final reaction with many of the compounds, especially upon incubation for a long time. *B. radiobacter* induced, in each case where good growth occurred, a strongly alkaline condition, probably due to the reduction of the nitrate. The acid reaction, induced by *P. tumefaciens* when ethyl or propyl alcohol was used, may result from the rapid rate of oxidation of the alcohol to acid in comparison with the slow release of the basic ions as the nitrate nitrogen was utilized. This may likewise account for the difference in reaction induced by *B. radiobacter* where these two alcohols were employed. The alkaline reaction induced with sodium formate, sodium acetate, and sodium succinate by *P. tumefaciens* may come through the accumulation of basic ions upon utilization of the organic radical, or upon absorption of the nitrate ion. As a general rule, the sugars and a few of the poly-hydroxy alcohols were better suited than the other materials to the growth of those two organisms, although some of the organic acids or their salts seemed readily available. The differences noted are worthy of further study in relation to the pathogenicity and nonpathogenicity of the two organisms.

NITRATE REDUCTION IN RELATION TO CARBON SOURCE

The nitrate reduction studies were made, using various sources of carbon in the nitrate medium previously described. Inoculation was made from slant cultures by means of a one millimeter wire loop and incubated at 28°C. Qualitative tests were made for nitrites with Trommsdorf's reagent, and for nitrates with a diphenylamine reagent. Results after three and six weeks incubation are reported, although observations in many cases were also made up to fourteen weeks. Table 3 shows a summary of these qualitative tests.

TABLE 3

*Summary of results of qualitative tests for nitrate and nitrite in media containing potassium nitrate with various carbon sources, and acted upon by Phytomonas tumefaciens or Bacillus radiobacter**

SOURCE OF CARBON	*P. tumefaciens* (9 STRAINS)		*B. radiobacter* (10 STRAINS)	
	Nitrate†	Nitrite†	Nitrate†	Nitrite†
Arabinose	+++	0	+++	0
Glucose	+++	0	0‡	0
Lactose	+++	0	+++	0
Sucrose	+++	0	+++	0
Raffinose	+++	0	0‡	0
Dextrin	+++	+	+++(0)	0
Salicin	+++	0	+++	0
Methyl	+++	0	+++	0
Ethyl	+++	+	+++§	0
Propyl	+++	0	+++	0
Butyl	+++	0	+++	0
Glycerol	+++	+	0	0
Mannitol	+++	+(0)	0‡	0
Adonitol	+++	+(0)	+++	0
Quercit	+++	0	+++	0
Dulcitol	+++	0	+++	0
Lactositol	+++	0	+++	0
Arabitol	+++	0	0‡	0
Sodium formate	+++	0	+++	0
Sodium acetate	+++	0	+++	+(0)
Sodium propionate	+++	++(+)	+++(++)§	++(0)
Butyric acid	+++	0	+++	0
Lactic acid	+++	++(+)	+++(0)	++(0)
Glycollic acid	+++	0	+++	0
Calcium gluconate	+++	+(0)	0‡	0
Stearic acid	+++	0	+++	0
Sodium oxalate	+++	0	+++	0
Sodium malonate	+++	0	+++	0
Sodium succinate	+++	+(0)	+++	0(++)
Malic acid	+++	+	−	+++
Tartaric acid	+++	0(++)**	+++	0(+)**
Sodium citrate	+++	0	+++	++(+++)

* The composition of the medium employed is given in the text. Tests for nitrate were made with diphenylamine reagent, and for nitrite with Trommsdorf's reagent. Controls tested showed nitrate but no nitrite. The results are given of tests made after three weeks. Similar tests were made after six weeks, but the results are omitted when they showed no change. Results after six weeks are shown in parenthesis when they differed from the set taken at three weeks.

† Tests recorded as follows: 0 = none, + = trace, ++ = moderate, +++ = strong, − = no determination made.

‡ Trace in a few cultures.

§ Nitrates disappeared from a few cultures at nine weeks.

** Test at nine weeks.

The nitrate completely disappeared from *B. radiobacter* cultures in three weeks when glucose, raffinose, glycerol, mannitol, arabitol, and calcium gluconate were employed as the carbon source. With dextrin and lactic acid the nitrate had completely disappeared in six weeks, and with sodium propionate or ethyl alcohol in nine weeks.

Nitrite was present at one period or another with *B. radiobacter* when sodium acetate, sodium propionate, lactic acid, sodium succinate, malic acid, tartaric acid and sodium citrate were used. Nitrites appeared with *B. radiobacter* more freely when the organic acids or their salts were used than with other sources of carbon. Nitrite was found with the crown-gall cultures where dextrin, ethyl alcohol, glycerol, mannitol, adonitol, sodium propionate, lactic acid, calcium gluconate, sodium succinate and malic acid were used as carbon sources. Where nitrite was present after three weeks it was not always present after six weeks, and vice versa. As a general rule the presence of nitrite with *P. tumefaciens* cultures was detected most often with the alcohols or the organic acids.

Daily qualitative tests for nitrate reduction were made upon media containing glycerol as the carbon source, and inoculated with *P. tumefaciens* or *B. radiobacter*. Either phenol red or meta-cresol purple was added as an indicator. In these trials, both with and without the indicator present, it was found that a crown-gall culture showing the presence of nitrite one day might not necessarily show it the following day. With *B. radiobacter* no nitrite was observed in the daily tests except in one instance where one culture showed a trace of nitrite on the sixth day, but none thereafter. In the majority of cases, with this medium, the nitrate disappeared between the seventeenth and nineteenth days from cultures of *B. radiobacter*. A change in reaction of the *B. radiobacter* cultures as shown by the indicator was first noticeable at nine days. The disappearance of nitrate without the appearance of nitrite is discussed following the quantitative determinations. The cultures incubated at 30°C. showed a more uniform disappearance of the nitrates than at room temperature.

QUANTITATIVE STUDIES ON NITRATE REDUCTION

Without peptone

Quantitative studies of nitrate reduction were also made. Arabinose, glucose, sucrose, and mannitol were used as carbon sources. Preparation of the media and inoculations were made as described earlier. The medium contained approximately 0.7 gram of nitrate nitrogen per liter. The cultures were incubated at two different temperatures, 25° to 27°C. and at 32°C. These two temperatures were used because Riker (1926) found that gall formation in tomato plants followed inoculations with the crown-gall organism at the lower but not at the higher temperature. Only the results obtained at 32°C. are reported as there was no material difference between these results and those obtained at 25° to 27°C. The analysis in certain cases was made in terms of both total nitrogen and nitrate nitrogen. The total nitrogen was determined by the Davisson-Parsons method (1919), and the nitrate nitrogen by the Gunning-Arnold method (1907). The organisms were grown in culture tubes containing 10 cc. of medium, and were introduced into a Kjeldahl flask, along with 90 cc. of distilled water for analysis. The results show (table 4) that nitrate had not completely disappeared in three to six weeks from the *B. radiobacter* cultures, but that much more had been lost than from the *P. tumefaciens* cultures. The crown-gall cultures, perhaps utilized the nitrate only in building bacterial protoplasm. The macroscopic evidence of growth with the two organisms was about the same.

With peptone

Further quantitative studies were made upon the disappearance of nitrate where 0.1 per cent Difco peptone was added to the medium previously used. This contained approximately 6.8 mgm. of nitrate nitrogen and approximately 8.4 mgm. of total nitrogen per 10 cc. *P. rhizogenes* was used along with the other two organisms. Only the single temperature of 32°C. was used.

In the presence of peptone, the nitrate was reduced more rapidly by *B. radiobacter* than where potassium nitrate was the only source of nitrogen. Nitrates disappeared (table 5) from the nitrate-peptone medium after six weeks incubation. A comparison of the total nitrogen lost from the tubes at six weeks, with that of the nitrate nitrogen at the start, showed that the lost nitrogen is approximately the same as that contained in the nitrate. A loss of nitrate with *P. rhizogenes* was shown in one instance (table 5), but is hardly beyond the limits of experimental error, so that one cannot consider that *P. rhizogenes* reduced nitrate. With *P. tumefaciens* there was some nitrate nitrogen loss, but the total

TABLE 4

*Loss of nitrate nitrogen from cultures of Phytomonas tumefaciens and Bacillus radiobacter, using media containing potassium nitrate and different sources of carbon**

CULTURE	WEEKS	LOSS OF NITRATE NITROGEN FROM 10 CC. WITH			
		Arabinose	Glucose	Sucrose	Mannitol
		mgm.	*mgm.*	*mgm.*	*mgm.*
P. tumefaciens	3	0.54	0.16		0.09
P. tumefaciens	6	0.62	0.30	0.53	0.89
B. radiobacter	3	0.63	0.73		1.03
B. radiobacter	6	3.03	3.60	2.74	1.79

* The composition of the media is given in the text.

TABLE 5

*Loss of nitrogen from cultures of Phytomonas tumefaciens, Phytomonas rhizogenes, and Bacillus radiobacter in media containing different sources of carbon, and with potassium nitrate and peptone present**

CULTURE	LOSS OF NITROGEN FROM 10 CC. WITH							
	Arabinose		Glucose		Sucrose		Mannitol	
	Nitrate	Total	Nitrate	Total	Nitrate	Total	Nitrate	Total
	mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
P. tumefaciens	0.20	0.30	0.72	0.05	0.62	0.10	0.06	0.15
P. rhizogenes	0.00	0.07	0.00	0.02	0.19	0.00	0.00	0.12
B. radiobacter	6.85	6.89	6.85	6.74	6.85	6.64	6.75	6.69

* Composition of the media is given in the text. Analysis was made after forty-two days incubation at 32°C. Nitrate was determined by Gunning-Arnold method, and total nitrogen was determined by the Davisson-Parsons method.

nitrogen loss in all cases but one, where arabinose was used as the carbohydrate, was very small. This indicates that the nitrate was not reduced to any extent beyond the point where nitrogen was utilized by the microörganism for bacterial protoplasm.

Qualitative tests showing nitrate present and nitrate absent in short time incubation periods cannot be taken as conclusive evidence that no nitrate reduction has occurred. The organism may reduce only a few molecules of the nitrate at any one time, but reduce them completely, in which case a qualitative test for nitrite would be of no value. However, when quantitative analysis was used as a measure of the loss of nitrate nitrogen and the loss of total nitrogen was obtained, such analyses showed that considerable nitrate nitrogen had disappeared with the cultures of *B. radiobacter*. If the nitrate was reduced to free nitrogen, it would have been lost from the solution rapidly. If the reduction proceeded to ammonia, this might have been lost as a gas from cultures which became alkaline, as with *B. radiobacter* in certain media.

TIME-POTENTIAL MEASUREMENTS

In view of the fact that in certain instances *B. radiobacter* reduced nitrates completely, which was not the case with *P. tumefaciens*, it was desirable to see if there was any difference between the oxidation-reduction potentials induced by the different organisms. This electrometric determination of the oxidation-reduction potentials of the systems studied was made with the use of the standard calomel half-cell and a vacuum-tube potentiometer as described by Allyn and Baldwin (1932). The culture tubes used were constructed with an enlarged end, in order to prevent the electrodes contacting the walls of the tube. These tubes were blown from eight-inch test tubes.

The electrodes used were made from platinum wire of No. 32 B. and S. gauge. Two electrodes, together with a saturated potassium-chloride agar bridge, were used per tube, and were sterilized by autoclaving. The two electrodes, together with a tube for the potassium-chloride agar bridge, and a larger glass tube drawn to a fine bore, through which the inoculum was added, were held together by wrapping with cotton which served as the cotton plug. These were suspended in the large test tube with the lower end of the tube for the potassium-chloride agar bridge placed in a smaller tube of potassium-chloride agar, while the lower ends of the electrodes were immersed in water between the walls of the two tubes.

The media used were (1) the glucose nitrate medium, (2) the glucose nitrate peptone medium used in the study of nitrate reduction, and (3) the glucose potato-extract medium used in the study of utilization of carbon sources. Fifty cubic centimeters of the medium were placed in the large test tubes, autoclaved for one hour at 15 pounds steam pressure and cooled at room temperature. Then the potassium-chloride agar bridge and electrodes were transferred to the tubes of culture medium.

Potentiometer readings were taken on the sterile media as soon as possible, usually at about one to one-and-one-half hours after the material was removed from the autoclave. Inoculations were made with 1 cc. of a suspension of the organism, standardized to a turbidity as previously mentioned. A control tube open to the air was used in addition to a control sealed from the air by means of a 3-inch vaseline plug. The cultures were incubated at approximately 25°C. Potentiometer readings were taken at quite frequent intervals for the first two days, but later were made two or three times per day.

The organisms commonly lowered the oxidation-reduction potential during their growth, as manifested by the potential-time measurements obtained (figs. 1, 2, and 3). The potential in the glucose nitrate medium was lowered very rapidly by *B. radiobacter*, as shown in figure 1. The medium, however, became more oxidized again. Two such trends were observed at about the eighth and sixteenth day, after which there was a gradual decrease in the potential to about the twenty-fifth day, to a value which was then maintained for approximately two weeks. In one instance with the glucose nitrate medium, it was noted that the potential again dropped suddenly after the fortieth day to Eh -0.214, where it was stabilized. These observations correlate with the nitrate reduction data where it was noted that in many

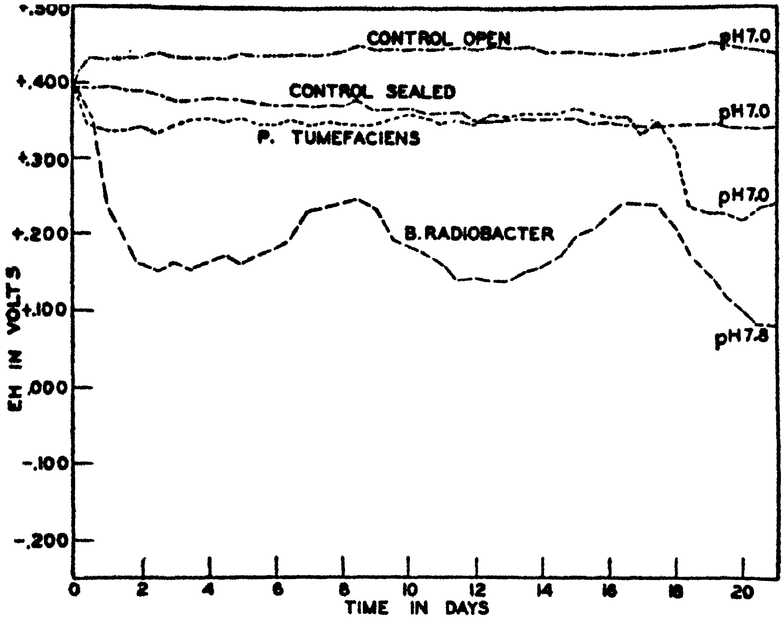

Fig. 1. Potential-time Curves of Glucose Nitrate Basal-salt Medium Inoculated with Phytomonas tumefaciens and Bacillus radiobacter

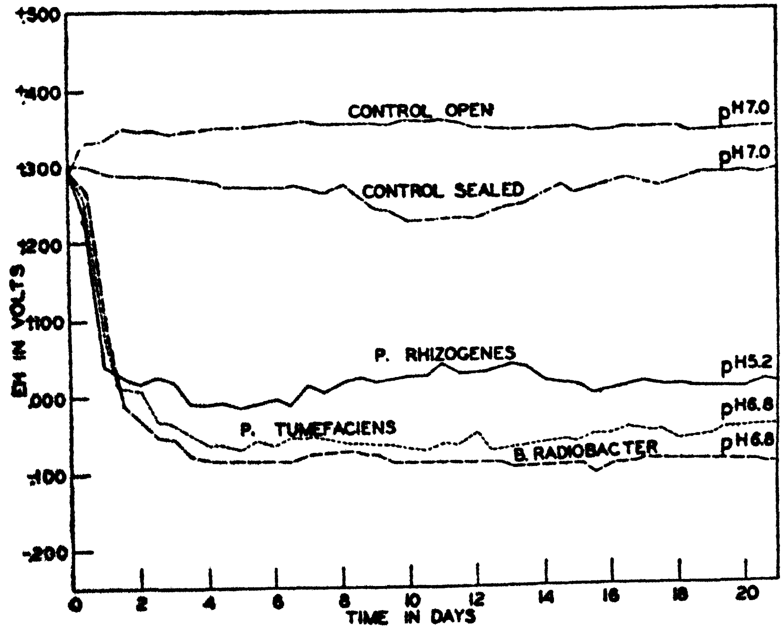

Fig. 2. Potential-time Curves of Glucose Peptone Basal-salt Medium Inoculated with Phytomonas tumefaciens, P. rhizogenes and Bacillus radiobacter

cases the nitrates disappeared at about the seventeenth to nineteenth day while in other cases this required a longer period. The reaction at twenty-one days was pH 7.8, and at forty-two days it was pH 8.8. The control tube was at pH 7.0. The potential was not lowered to any extent by *P. tumefaciens* in the same medium, figure 1. There occurred a period of a few days in which the oxidation-reduction potential was somewhat reduced, but as a general rule the potential was not much lower than with the uninoculated and sealed tube. On the forty-fourth day the potential dropped to Eh +0.228, which is approximately the same potential reached on the eighteenth day, but which had been maintained only for a few days. The pH was at 7.0.

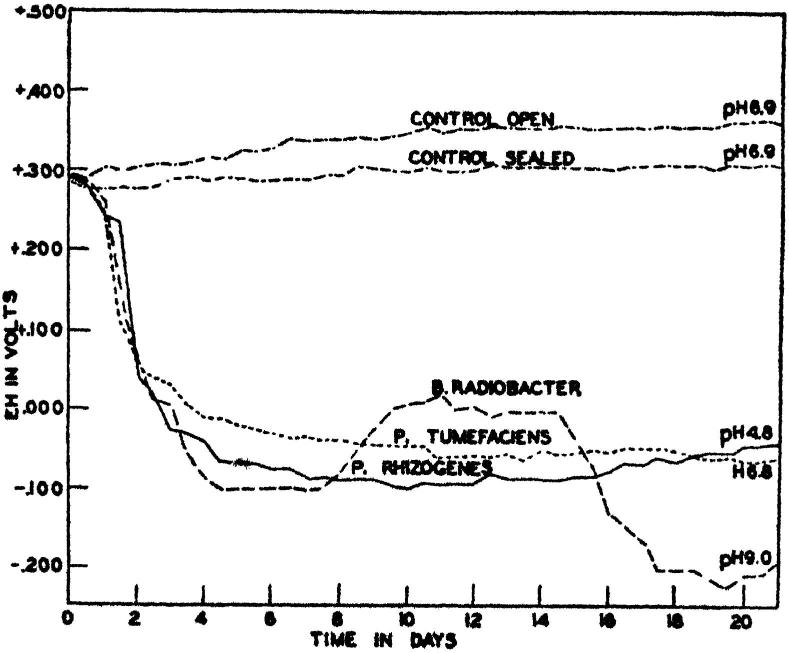

FIG. 3. POTENTIAL-TIME CURVES OF GLUCOSE PEPTONE NITRATE BASAL MEDIUM INOCULATED WITH PHYTOMONAS TUMEFACIENS, P. RHIZOGENES AND BACILLUS RADIOBACTER

With the glucose peptone medium the lowest potential was again produced by *B. radiobacter*. It may be noted, figure 2, that *P. tumefaciens* and *P. rhizogenes* although not producing quite as low a potential as *B. radiobacter*, held the potential rather uniformly parallel to that of *B. radiobacter*.

A decided drop in potential was produced by each of three organisms in the glucose nitrate medium to which 0.2 per cent peptone had been added (fig. 3), with *B. radiobacter* producing the lowest potential. After a few more days the potential produced by *B. radiobacter* was higher than before and again after another week dropped suddenly to Eh −0 200. This potential was not reached in the nitrate medium until the fortieth day. Qualitative tests for nitrates, made on the thirtieth day, showed nitrates to be present with the crown-gall and hairy-root cultures, but absent with the *B. radiobacter* cultures. This again helps to explain the findings in the quantitative nitrate reduction studies where it was shown, tables 4 and 5, that the addition of peptone markedly increased the rate of nitrate reduction. In this medium *P. tumefaciens* and *P. rhizogenes* did not produce as low a potential as *B. radiobacter*. *P. rhizogenes* produced a slightly lower potential than the crown-gall organism for about a two-week period, after which it became less reduced.

The potential in the glucose potato extract medium was lowered to about the same extent by all three organisms. It was noted, however (fig. 4) that the potential of the controls, both open and closed, was much lower at the start of the experiment than with the other media used. The potential of the open tube decreased for about one day; after which it increased gradually for about ten days. The closed control, however, showed a rather rapid drop in potential on the first day which was about the same as for the open control. The potential then decreased slowly to about the sixth day; after which it seemed to be stabilized at about an Eh 0.0. All of the cultures grew more quickly in the glucose potato-extract medium than in any of the other media. This was especially noticeable in connection with *P. rhizogenes*.

If one compares the potential of the controls in the various media, it is noted that there is no great difference between the medium exposed to the air and the same medium sealed from the air, except in the case of the glucose potato-extract medium. In this instance, as noted previously, the potentials were about the same for the first day, after which they became more separated.

DISCUSSION

The results of work on the utilization of nitrogen from different sources showed that considerable difference existed between *P. rhizogenes* on the one hand, and *P. tumefaciens* and *B. radiobacter* on the other. This was more marked in the basal-salt media with various carbohydrates, where an inorganic source was supplied as nitrate nitrogen. Under the conditions used in this study, *P. rhizogenes* was much more limited in its utilization of nitrogenous compounds than was either *P. tumefaciens* or *B. radiobacter*.

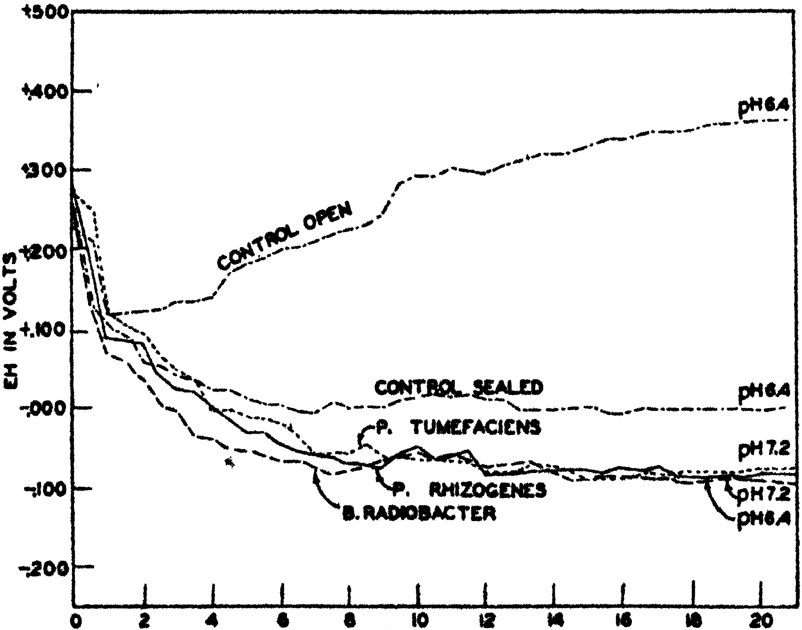

FIG. 4. POTENTIAL-TIME CURVES OF GLUCOSE POTATO-EXTRACT MEDIUM INOCULATED WITH PHYTOMONAS TUMEFACIENS, P. RHIZOGENES AND BACILLUS RADIOBACTER

P. rhizogenes was unable to utilize to any extent the inorganic sources of nitrogen. The inability of *P. rhizogenes* to produce growth in a nitrate medium might come, not alone from the inability to utilize nitrates, but from other factors among which should be mentioned (1) an unfavorable oxidation-reduction potential, and (2) the lack of growth-stimulating substances.

Work by Allyn and Baldwin (1932), Dubos (1929), Webster (1925), Wright (1929), and others, in which it has been shown that organisms have an optimum oxidation-reduction potential for growth, indicates that the somewhat similar results obtained in this study may be concerned with the ability of the organism to utilize nitrate nitrogen. The failure of *P. rhizogenes* to utilize nitrate nitrogen in a 0.5 per cent nitrate medium, and its ability to utilize nitrate nitrogen to a slight extent where a reducing agent was added, gives further strength to this suggestion. In none of our studies with the use of a reducing agent has good growth been obtained with *P. rhizogenes* where nitrate nitrogen has been used as the only source of nitrogen.

The fact that *P. rhizogenes* produced its best growth in a glucose, potato-extract medium may result from the lower oxidation-reduction potential of the medium at the beginning. It may, on the other hand, be explained by the presence of some growth-stimulating substance. Other workers have found that the addition of very small amounts of certain complex substances to the medium enabled certain microörganisms to utilize otherwise unavailable nitrogen sources. This likewise may be the reason for the good growth produced by the organisms in media containing peptone.

In the study of the utilization of nitrogen sources as shown by growth, *P. tumefaciens* and *B. radiobacter* each utilized nitrogen-containing compounds to about the same extent. The difference in nitrate reduction is worthy of further consideration in relation to the pathogenicity and nonpathogenicity respectively of the two organisms.

When the various sugars, glucosides, and alcohols were used as a carbon source, *B. radiobacter* produced an alkaline reaction in all cases where it made satisfactory growth except with propyl alcohol. The alkaline reaction was undoubtedly because of the fact that potassium nitrate was reduced and the basic ions remained in excess. With butyl alcohol, only a trace of growth resulted and no change in reaction was produced. When propyl alcohol was employed *B. radiobacter* and *P. tumefaciens* both produced good growth and an acid reaction. This alcohol may be oxidized to an acid rapidly enough to overcome the alkalinity resulting from nitrate reduction. The utilization of ethyl alcohol with an acid reaction by *P. tumefaciens* and an alkaline reaction by *B. radiobacter* is noteworthy.

The basic reaction resulting from the utilization of the salts of the organic acids may have been in part because of the more or less complete utilization of the acid radical with the basic ions remaining. An acid reaction resulted in most instances where carbohydrates were utilized by *P. rhizogenes*, while in the same instances very little if any acid was formed by *P. tumefaciens*.

The reduction of nitrate by the various organisms, where nitrate was used in the media as a nitrogen source, is in part correlated with the potentials obtained. *B. radiobacter* induced the lowest potential. Likewise *B. radiobacter* reduced nitrates to the greater extent. The time of nitrate reduction by *B. radiobacter* also corresponded closely to the periods of rapid drop in potential.

SUMMARY

A comparison has been made of certain physiological characters of *P. tumefaciens*, *P. rhizogenes*, and *B. radiobacter* with emphasis on their nitrogen and carbon metabolism.

In these studies cultures obtained from both colony isolation and single-cell origin were employed.

Organic and inorganic compounds containing nitrogen were utilized, as shown by growth, as sources of nitrogen by *P. tumefaciens* and *B. radiobacter*. Amino acids were utilized to about the same extent by *P. tumefaciens* and *B. radiobacter*. Amino acids singly or in a mixture, and inorganic sources of nitrogen were utilized little if at all by *P. rhizogenes*. Nitrate nitrogen was utilized by *P. rhizogenes* to a very limited extent where a reducing agent was employed.

Nitrates were reduced to a very small extent by *P. tumefaciens*, but completely by *B. radiobacter* when certain carbon sources were employed. They were reduced more quickly by *B. radiobacter* in a medium containing both nitrate and peptone, than in a medium containing nitrate but no organic nitrogen compounds.

Glucose potato-extract was one of the most favorable media for the growth of *P. rhizogenes.*

Where various carbohydrates, glucosides, alcohols, and organic acids or their salts were utilized by *P. tumefaciens* and *B. radiobacter* they were used to about the same extent as indicated by growth, but with some differences in final reaction.

All three types of organisms when grown in a potato-extract glucose medium reduced the potential of the medium to about the same extent, whereas when grown in a glucose nitrate medium, in a glucose nitrate peptone medium, and a glucose peptone medium, they showed considerable difference with respect to the potentials induced.

Oxidation-reduction potentials determined over a period of time help to account for quicker reduction of nitrate when peptone was added to the medium. Literature will be cited at the end of Part II.

STUDIES ON CERTAIN PHYSIOLOGICAL CHARACTERS OF PHYTOMONAS TUMEFACIENS, PHYTOMONAS RHIZOGENES AND BACILLUS RADIOBACTER

PART II[1]

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INTRODUCTION

Our study of the physiology of the several bacteria considered in Part I; viz., (1) *Phytomonas tumefaciens*, (2) *Phytomonas rhizogenes* and (3) *Bacillus radiobacter* has been continued. Since any important variations in the physiology of these organisms might have an important bearing upon the reactions they induce in plant tissue, an attempt was made to discover any basic differences in their activity.

The present investigation has been concerned primarily with three lines of study. An attempt has been made (1) to extend available information on the physiological and pathogenic properties of the organisms and to observe any correlation between physiological behavior and pathogenicity; (2) to study variations in the several organisms as they might be induced by continuous cultivation in certain media under favorable and unfavorable conditions and (3) to determine the effect of successive passages through plants upon the physiological characteristics and virulence of the crown-gall organism.

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CULTURES USED

The purity of the bacterial strains employed was insured, as far as possible with available technic, by employing only cultures grown from individual bacterial cells. The method employed in isolating and culturing these single bacterial cells has been described by Wright, Hendrickson and Riker (1930).

The parent cultures employed in these studies were isolated from various hosts secured from widely separated regions.

The crown-gall cultures used in this study were secured as follows: A black-raspberry gall grown in Iowa in 1924 was the source of culture T5 from which seven single-cell cultures were secured. An almond gall grown in California in 1913 was the source of culture T2 from which four single-cell cultures were isolated. A walnut gall grown in California in 1926 was the source of T37 from which three single-cell cultures were obtained. Culture T3 was secured in 1916 from incense cedar and was the source of four single-cell cultures used. The writers secured some of these cultures through the courtesy of C. O. Smith and J. H. Muncie.

The hairy-root cultures were all obtained from apple. Hairy-root developments in Arkansas and Michigan in 1926 were the sources of cultures T32 and T34, respectively, from which five and four single-cell cultures were isolated. Hairy-root overgrowths secured in Wisconsin in 1925, 1926, and 1926 were the sources, respectively, of cultures T38, T48, and T49 from which three, one, and five single-cell cultures were isolated.

From radiobacter cultures R3, R11, and R16, secured from the United States Department of Agriculture, two, three, and two single-cell cultures respectively, were obtained.

A summary of the cultures employed shows that 43 single-cell strains were obtained from 4 crown-gall, 5 hairy-root, and 3 radiobacter cultures. These single-cell cultures with their parent cultures, were employed in all the trials unless otherwise noted. Earlier trials of the pathogenicity of the parent cultures have been recorded by Riker *et al.* (1930).

Physiological tests were employed with these cultures which showed in part their identity. Among these tests were growth in litmus milk, calcium glycerol phosphate agar plates, peptone broth, and glycerol phenol-red broth as described in Part I. Sugar fermentation was determined in the basal medium, described by Riker *et al.* (1930) and Wright, Hendrickson and Riker (1930), to which was added 0.5 per cent lactose or glucose. In all cases the bacteria in question gave typical responses except culture T2 and T37 and their single-cell progeny. The physiology of these cultures will be described later.

Plant inoculations into tomato and apple were also employed as further evidence of the identity of these cultures. Young tomato plants were used first, because of the rapidity with which the galls form, and the ease with which the plant could be obtained. The tomato plants after puncture inoculation were placed at about 70°F. in the greenhouse for four to six weeks. Apple-graft inoculations were also made with these cultures in the field and results were taken after two to three months. The series of inoculations on both tomato and apple were performed on 2 plants (5 inoculations per plant) and were repeated three separate times.

The results secured, in accord with those noted by Riker *et al.* (1930), showed that *P. tumefaciens* produced crown-gall on both tomato and apple, *P. rhizogenes* produced little or no reaction on tomato but hairy-root on apple and *B. radiobacter* no reaction on either tomato or apple. With the exception of cultures T2–1b and T37–2b and their single-cell progeny, discussed in detail later, the characterization of the cultures by their physiological reactions and their plant pathogenicity agreed in each case.

PHYSIOLOGICAL DIFFERENCES

Additional physiological differences between these organisms were sought by employing modifications of media already tried and by using other types of substrata.

Reduction of selenium salts

Transfers to a sodium-selenite medium, on the basis of earlier reports (Klett, 1900; Levine, 1925; Scheurlen, 1900; and Waksman, 1927), appeared promising for the differentiation of the bacteria studied.

Sodium selenite yeast-water glucose agar medium was prepared as follows: glucose, 5.0 grams; sodium selenite, 0.1 gram; agar, 15.0 grams; and 1000 cc. of 1 per cent yeast water. Transfers of the various cultures were made to agar slants and incubated at 20°C. for four days. The hairy-root organisms did not grow while the crown-gall and radiobacter cultures produced abundant growth with a distinct red color in the streak, because of the presence of free selenium. The sodium selenite apparently was much more toxic to the hairy-root than to the crown-gall or radiobacter organisms.

Absorption of dyes

Since various dyes have been useful (Kellerman, 1913; Riker *et al.*, 1930) in differential studies, fifteen dyes were used in preliminary trials at concentrations of 1 to 10,000 and 1 to 20,000 with yeast-water mannitol agar. The various dyes used were aniline blue, acid fuchsin, basic fuchsin, Bismark brown, crystal violet, dahlia eosin, fluorescein, orcein, methyl orange, night blue, nigrosin, rosanilin, safranin, and thionin. The only dyes that showed differential absorption or bacteriostatic effect were dahlia, thionin, Bismark brown and aniline blue. At a concentration of 1 to 10,000, dahlia retarded the growth of both the crown-gall and hairy-root organisms while Bismark brown and thionin retarded the growth of the hairy-root cultures. Thionin was the most effective of these three dyes in the inhibition of growth. In this preliminary work it was observed that the crown-gall organism absorbed the aniline blue dye, while the hairy-root and radiobacter cultures did not. Since aniline blue seemed the most promising of these dyes, it has received more detailed attention.

Water suspensions of these organisms were streaked on yeast-water mannitol aniline-blue agar plates and then incubated at room temperature. The medium had the following composition: mannitol, 5.0 grams; magnesium sulfate, 0.2 gram; dipotassium phosphate, 0.2 gram; sodium chloride, 0.2 gram; calcium sulfate, 0.1 gram; aniline blue, 0.1 gram; agar, 15.0 grams; 100 cc. of 10 per cent yeast water; and 900 cc. of distilled water; adjusted to pH 7.0. All three types of cultures grew abundantly on the medium and they differed only in their ability to absorb the aniline blue. The color absorbed by the crown-gall organism was a distinct and characteristic blue.

The concentration of aniline blue within the limits employed, 1 to 1000, 1 to 10,000 and 1 to 20,000, seemed to make little difference.. At the highest dilution there was some absorption of the blue dye, but the streak did not become so intensely blue as at the 1 to 10,000 dilution which was used in all later studies. At a concentration of 1 to 1000 the medium was so blue that the absorption of blue color by the organism was not readily ascertained. At none of these concentrations did the hairy-root or radiobacter cultures absorb the blue dye. All three groups of organisms grew equally well at the various concentrations used.

The hydrogen-ion concentration of the medium, between pH 4.4 and 8.0, had no effect upon the absorption of the blue dye by the crown-gall organism. In this range the crown-gall organism absorbed the blue dye while the radiobacter and the hairy-root cultures did not.

The composition of the medium influenced the absorption of aniline blue by the crown-gall organism. Growth of the organism on potato glucose agar plus aniline blue, 1 to 10,000, showed no absorption of the blue color by either crown-gall, hairy-root or radiobacter cultures. The crown-gall cultures were slow in absorbing the blue color in Patel's (1926) medium when aniline blue was substituted for crystal violet. They did absorb and retain the blue dye, however, after seven to ten days.

Crystal violet added in concentrations of 1 to 20,000, 1 to 40,000, 1 to 50,000 and 1 to 100,000 did not prevent the crown-gall bacteria from absorbing the blue dye. However, at the greater concentrations of crystal violet there seemed to be slightly less dye absorbed. The radiobacter and hairy-root cultures did not absorb the blue dye in any of these tests.

Three different samples of aniline blue were tested and the blue color was absorbed from each by the crown-gall organism. Water solutions of two samples of dye exhibited a purplish color. This coloration was apparently due to two constituents, a blue and a violet one. A third sample was apparently more highly purified as it appeared blue without any tinge of purple. All samples served as hydrogen-ion indicators. Two of the samples never became entirely colorless on the alkaline side while the other sample was colorless at pH 7.0. On a medium adjusted to this hydrogen-ion concentration and containing the latter sample of dye, the growth of the crown-gall organism appeared as a clear blue streak on a white background. Evidently the hydrogen-ion concentration of the medium did not interfere with the absorption of the dye and the hydrogen-ion concentration of the bacterial mass was acid enough to restore the blue color. This dye absorption was useful in isolating the bacteria from galls.

Isolation medium

A selective medium is desirable for the isolation of the crown-gall organism. Surface sterilization of crown galls with mercuric chloride is not commonly satisfactory because the organisms are very sensitive to this chemical. Diseased tissue frequently contains a variety of saprophytes which develop rapidly on agar plates. Soil particles adhering to the malformation cause considerable contamination. These factors and the close relationship between the crown-gall organism and many other bacteria found in the soil made it desirable to have a selective medium for the isolation of the crown-gall organism. Patel's (1926) medium has been commonly and successfully used.

Aniline blue in yeast-water mannitol medium was such a distinct aid in the isolation of the crown-gall organism that it has been commonly used. When 0.1 gram of aniline blue per liter was added to yeast-water mannitol agar the crown-gall organism was readily isolated from diseased tissue and even from macerated gall tissue to which garden soil had been added. This dye inhibited most of the molds. The crown-gall organism was usually present in great abundance and was readily recognized by the blue color of the colonies. However, many types of colonies of other organisms were also present, some of which appeared purple or lavender but none showed the distinctive blue color. Doubtless other bacteria do absorb this dye with the same color character, but so far they have not been encountered in crown-gall tissue. Since crown-gall organisms may exist which do not absorb aniline blue, as is shown later in this paper on the selection of sectors from colonies on yeast-water mannitol aniline blue agar, this medium has perhaps certain limitations as well as advantages.

The absorption of aniline blue by the crown-gall organism has been studied to test whether or not the dye might lower the virulence of the culture. The crown-gall organism was allowed to grow upon yeast-water mannitol aniline blue medium for six weeks and then was inoculated into young tomato plants. Typical crown gall was formed in each case. The growth upon the medium seemed to have no effect upon either the virulence or the longevity of the organism.

Growth in relation to oxidation-reduction potentials

Growth in a highly oxidized medium employed showed physiological differences between hairy-root bacteria on one hand and the crown-gall and radiobacter organisms on the other. Potassium nitrate mannitol agar medium was used which had the following composition: mannitol, 10.0 grams; potassium nitrate, 0.5 gram; dipotassium phosphate, 0.5 gram; sodium chloride, 0.2 gram; agar, 15.0 grams; and distilled water, 1000 cc. It was adjusted to pH 7.0. Transfers of a considerable number of bacteria from each culture, respectively, were made into shake agar cultures that were incubated at room temperature. They were examined after four and ten days. The crown-gall and radiobacter organisms produced abundant growth at the surface, while the hairy-root cultures formed a zone of growth from the surface down to a depth of one-fourth inch. When the oxidation-reduction potential of the medium was increased by the addition of 0.1 gram of potassium permanganate per liter the crown-gall and radiobacter cultures still grew abundantly at the surface, while the hairy-root cultures either failed to grow or produced only a slight growth below the surface. Sagen *et al.* (Part I of this paper) have found that the hairy-root organisms did not grow in liquid potassium-nitrate mannitol medium, as they appeared to be unable to utilize potassium nitrate as a source of nitrogen. It is probable that the hairy-root organisms grew in these agar shake cultures because of the presence of impurities in the agar. The addition of potassium permanganate apparently prevented growth of the hairy-root organism because of the highly oxidized condition it produced.

Growth in ferric-ammonium-citrate medium, another highly oxidized substratum, also showed physiological differences between hairy-root bacteria on one hand and the crown-gall and radiobacter organisms on the other hand. The medium consisted of 10.0 grams ferric-ammonium citrate, 0.5 gram magnesium sulfate, 0.5 gram dipotassium phosphate, 0.2 gram calcium chloride and 1,000 cc. of distilled water, adjusted to pH 7.0. The crown-gall and radiobacter cultures both produced a distinct reddish-brown pellicle at the surface and a reddish-brown coloration in the medium, apparently because of the formation of ferric hydroxide. The hairy-root culture frequently did not produce any growth; and where growth did appear there was no reddish-brown discoloration.

Similar differentiation was secured when this medium was used for agar shake or slant cultures. In agar shake cultures the crown-gall and radiobacter cultures grew at the surface and produced a zone of reddish-brown color in the upper portion of the medium. The hairy-root cultures sometimes grew to a slight extent below the surface but did not produce any reddish-brown color zone. Similarly, on agar slants the crown-gall and radiobacter cultures grew abundantly and produced reddish-brown coloration in the medium while the hairy-root cultures either failed to grow or grew slightly without the reddish-brown color.

The amount of ferric ammonium citrate may be varied from five to twenty-five grams per liter; but 10 grams proved the best proportion. With a concentration of 2.5 grams (in liquid medium) the hairy-root organisms produced more visible growth, so that it was difficult to separate the three organisms during the early stages of growth. But after ten days the crown-gall and radiobacter organisms could be readily distinguished. The addition of 0.1 gram potassium permanganate to this medium containing 10.0 grams ferric ammonium citrate was usually employed in routine diagnosis of the organisms as it inhibited the growth of the hairy-root organisms even more definitely.

Oxidation-reduction potentials induced by growth

The oxidation-reduction potential of the medium was changed by the growth of the organisms. Preliminary studies indicated that the crown-gall (pathogenic culture, A1, and nonpathogenic culture, A6–6) and radiobacter organisms were slightly more active in inducing low oxidation-reduction potentials than the hairy-root organisms. These studies were made by determining the E.M.F. produced by the organisms in liquid medium as described by Allyn and Baldwin (1932).

The ferric ammonium citrate medium used was highly oxidized and well buffered. The Eh of the medium was about +450 millivolts (in terms of the hydrogen electrode) in the open control; the sealed control possessed a somewhat lower potential (about 100 millivolts) brought about by the exclusion of atmospheric oxygen.

There was no significant difference between the crown-gall (pathogenic, A1 and nonpathogenic, A6–6) and the radiobacter cultures from the standpoint of the oxidation-reduction potentials produced. These cultures reduced the medium quite rapidly at first and then more gradually, always remaining within a few millivolts of each other during the entire experiment, and never reducing the potential below zero under the conditions of these experiments. The formation of the brown pellicle could commonly be predicted by noting the E.M.F. produced. This indicated, in accord with Allyn and Baldwin (1932), that the pellicle formation of the crown-gall and radiobacter organism was associated with the oxidation-reduction potential of the medium.

The hairy-root organism reduced the oxidation-reduction potential as rapidly as the crown-gall and radiobacter organism during the first few days of growth. However, the potential soon rose and continued to rise gradually so that after 18 days it was slightly below that of the open control. Apparently the organism was able to initiate growth in the medium but was unable to continue growing; and subsequently the medium was reoxidized by atmospheric oxygen.

There was only a slight change in the hydrogen-ion concentration because of the growth of the organisms, and the changes in potential were largely owing to an actual increase in reduction intensity.

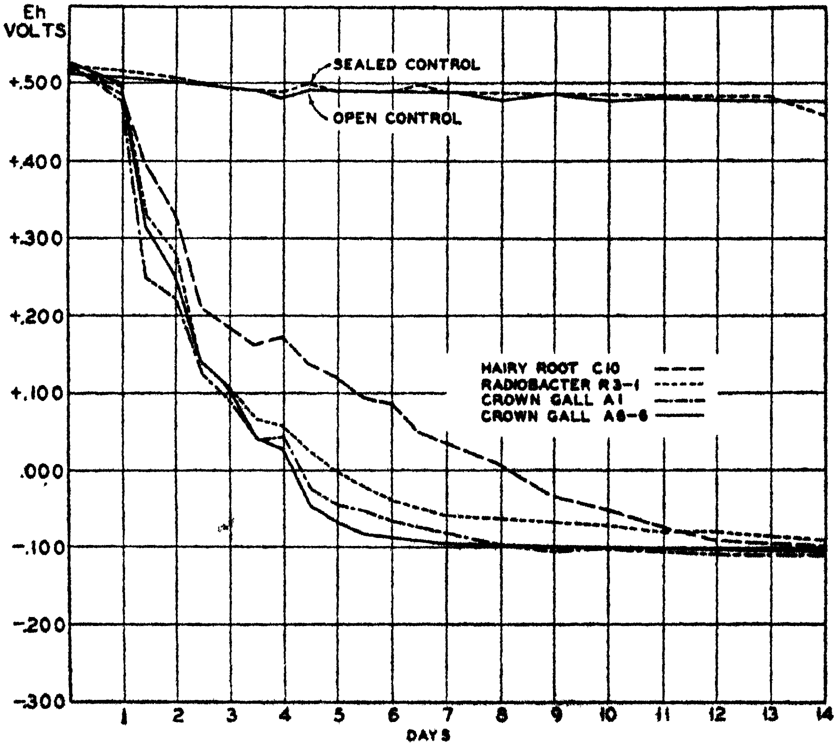

Fig. 1. Oxidation-reduction Potentials (in Terms of the Hydrogen Electrode) of Sealed Controls, Open Controls, and Cultures of Given Organisms during Two Weeks in Yeast-water Mannitol Aniline-blue Medium

After 31 days the results were almost identical with those at 14 days. The pH at the beginning was 6.3. After 31 days it was as follows: open control, 6.3; closed control, 6.3; hairy-root culture, 6.6; radiobacter, 6.3; crown gall A-1, 6.3; and crown gall A6-6 (nonpathogenic), 6.2.

The change in the oxidation-reduction potentials induced by the organisms in yeast-water mannitol aniline-blue medium, which was very favorable for the growth of all these organisms, was more marked than that mentioned above. The crown-gall (pathogenic and nonpathogenic) and radiobacter organisms reduced the medium very rapidly, reaching a potential after 5 to 7 days of approximately −100 millivolts which was maintained with very little change for a month (to the end of the experiment). The potentials induced by these organisms remained within a few millivolts of each other during the entire experiment (fig. 1).

In this medium the hairy-root organisms reduced the oxidation-reduction potential but with more of a lag than in the case of the crown-gall and radiobacter organisms. However, after the 14th day the oxidation-reduction potential induced by the hairy-root organism was essentially the same as that induced by the others.

ARTIFICIALLY INDUCED VARIATIONS

Many variations in *Phytomonas tumefaciens* have been observed since it was first isolated and described by Smith and Townsend (1907). Smith, Brown and Townsend (1911) saw variations in the organisms isolated from different hosts. The similarity between certain nonpathogenic organisms and the crown-gall organism has been observed by Brown and Leonard (1932). Patel (1929) noted that the two types were similar in their morphological and physiological characters and could be separated only by their pathogenicity. Stapp (1927) and other workers have recognized widely divergent strains of the crown-gall organism and report that various strains of *P. tumefaciens* may be separated from one another by agglutination. Rough and smooth forms of the organisms have been secured by Link and Hull (1927), Israilsky (1929), Muncie and Patel (1930). Israilsky and Starygin (1930) in continuing their studies upon the rough form, observed that the smooth and rough type of *P. tumefaciens* differed only slightly in several biochemical characteristics. They were inclined to assume that they were dealing with the intermediate O form, as they were able to observe by miscoscopical examination a whole series of transitory forms, and also because on plating, the rough form produced a constant percentage of smooth forms. This suggests that the organism readily undergoes dissociation and an attempt to induce variation in the culture by artificial means might be readily accomplished. However, it is scarcely necessary to mention that reports of variability in cultures, whose original purity might be in question, cannot be accepted without reservation.

Attempts to induce variation in culture by repeated transfer on artificial media

The uniformity in physiological characters among the single-cell cultures of each organism of the collection of cultures gave opportunity to study the possibility of inducing variation by cultural conditions and to observe the conditions under which variation occurred. Slight variations in culture had been previously observed in the streaks on calcium-glycerol phosphate plates. Some of the streaks showed sectors, and others, secondary colonies. These observations made attempts to induce variation appear promising.

Cultures and media employed. The procedure in these studies included all the single-cell cultures and their parent cultures, the histories of which have been previously described. These were carried with monthly transfers on six different media, selected because they had a wide range of carbohydrate-nitrogen ratios, pH values and oxidation-reduction potentials. All media were incubated at room temperature except nutrient gelatin which was incubated at 18° to 20°C. Three to four monthly transfers were made.

The following media were used: (1) Malt gelatin broth contained 10.0 grams gelatin, 10.0 grams malt extract, and 1000 cc. of distilled water. (2) Yeast-water glucose agar contained 5.0 grams glucose, 15.0 grams agar and 1000 cc. of 10 per cent yeast water. (3) Nutrient broth was composed of 5.0 grams peptone, 3.0 grams beef extract, and 1000 cc. of distilled water and was used at hydrogen-ion concentrations of pH 4.4, 7.0 and 8.0. (4) Nutrient gelatin was prepared by adding 150.0 grams of sheet gelatin to nutrient broth. (5) Peptone broth contained 5.0 grams peptone, 0.2 gram dipotassium phosphate, 0.2 gram sodium chloride, 0.1 gram calcium chloride and 1000 cc. distilled water. (6) Potassium-nitrate mannitol agar contained 10.0

grams mannitol, 0.5 gram potassium nitrate, 0.5 gram dipotassium phosphate, 0.2 gram magnesium sulfate, 0.2 gram sodium chloride, 15.0 grams agar and 1000 cc. distilled water. All of these various media were adjusted to pH 7.0 unless otherwise specified.

No real changes in the bacteria were found following growth on these media. There were indications that the cultures showed slightly atypical reactions on these various media after three to four monthly transfers, but when the cultures which showed slight variations were tested on various diagnostic media such as potassium-nitrate mannitol agar shake cultures, sodium-selenite agar slants, litmus milk, calcium-glycerol-phosphate agar plates and by inoculation into tomato plants, no change in the organisms was detected. Consequently the large amount of data accumulated in these studies is omitted.

The failure of this procedure might perhaps result from the fact that variation often takes place within a culture by the formation of aberrant organisms. These organisms might be relatively few in number in the culture or in various parts of the culture; so that when transfers were made to new media by the ordinary method the organisms which were transferred were predominantly of the normal type. Likewise, the medium might not be favorable for the growth of the aberrant organism which was consequently overgrown by the normal type. To overcome this difficulty, plating of the cultures was employed in the hope that any aberrant strains which appeared would have an opportunity to develop into colonies. Better success was secured with this technic.

Selection of variants by the plating technic. An avirulent culture of *Phytomonas tumefaciens* was sought by making colony isolations from a single-cell stock culture. Dilution plates of various single-cell cultures of the crown-gall bacteria were made on yeast-water mannitol agar. These cultures had been carried on this medium since their isolation. Thirty well isolated colonies from each culture were picked and transferred to new yeast-water mannitol agar slants. They were then inoculated into young tomato and sedum plants and tested on various physiological media. From over one hundred isolated colonies only one failed to produce galls.

This one and all the others reacted like typical crown-gall cultures on the diagnostic physiological media. The nonpathogenic culture was isolated from culture A6, a single-cell crown-gall culture. Many attempts to induce gall formation on tomato and sedum plants in different trials during the following year failed while the controls produced galls. From this culture sixteen colonies were isolated and then checked for physiological behavior and pathogenicity. They all reacted like typical crown-gall cultures on the physiological media, but failed to induce galls. Thus, a nonpathogenic crown-gall culture which reacts physiologically like the virulent crown-gall organism has been isolated. Since the parent culture was of single-cell origin, there is definite indication that the culture has undergone variation during cultivation on media.

Aniline blue sectors. Crown-gall cultures were studied for their ability to absorb aniline blue as described earlier in this paper. Colorless sectors in the blue streak on yeast-water mannitol aniline-blue medium had been observed in some of the cultures of the crown-gall organism. An effort was made to determine any correlation between this character and virulence. Organisms from the colorless sectors of various single-cell cultures were isolated and transferred to agar slants. After repeated trials 2 cultures were secured which showed no blue dye absorption. The physiological behavior on culture media and pathogenicity of these cultures was then determined. They both reacted like typical crown-gall cultures on the differential physiological media mentioned earlier and induced galls on young tomato plants. Thus, virulent crown-gall cultures were obtained which did not react typically on the yeast-water mannitol aniline-blue medium.

The effect of successive plant passages upon the physiological characteristics and virulence of the crown-gall organism

The effect of successive passages through plants was studied with 3 cultures: viz., A5 and A6 which are single-cell isolations of T5fff1 (itself a single-cell isolation of T5), and T3-1c-1, which is a single-cell culture of T3-1c. The single-cell and parent cultures in previous studies always produced galls on tomato plants and showed the typical physiological reactions of crown-gall organisms on the differential media mentioned earlier.

Young tomato plants were used for inoculations in the plant passage work. When they were about four to six inches in height, they were puncture-inoculated three times with the crown-gall organism from a forty-eight-hour-old agar slant of the culture. Duplicate plants were inoculated with each organism and allowed to develop for five to eight weeks. During this time large galls were formed.

After the development of the galls a thin outer layer of tissue was removed from the gall with a sterile scalpel and discarded. The remainder of the gall was removed and placed in a sterile Petri dish. Material from different galls of the duplicate plants were mixed and about five cubic centimeters of sterile distilled water were added and the gall macerated. Small pieces of sterile absorbent cotton were moistened in this liquid and placed in each of the three punctures in the stems of two new plants. Controls were carried in each series by making sterile punctures in the stems of other plants. After two or three days the absorbent cotton was removed from the punctures. At each transfer other plants were inoculated from agar slants of the original culture.

Organisms were isolated from a gall each time it was transplanted into a new plant. After suitable incubation, ten colonies from these isolations which appeared to be the crown-gall organism were transferred to yeast-water mannitol agar slants, for further study.

The plan and results of successive inoculations in different plants are illustrated in figure 2. In this manner the organisms were continually kept in the host tissue without growth *in vitro*, and representatives were secured in culture after each plant passage. This method of inoculation and isolation was performed with each of the three cultures used in the plant passage work.

An examination of the material showed no appreciable difference in the size of the galls produced on the different plants of each experiment, which indicates that plant passage had no apparent effect upon the virulence of the organism. In four instances noted in figure 2 galls did not form on the plants inocu-

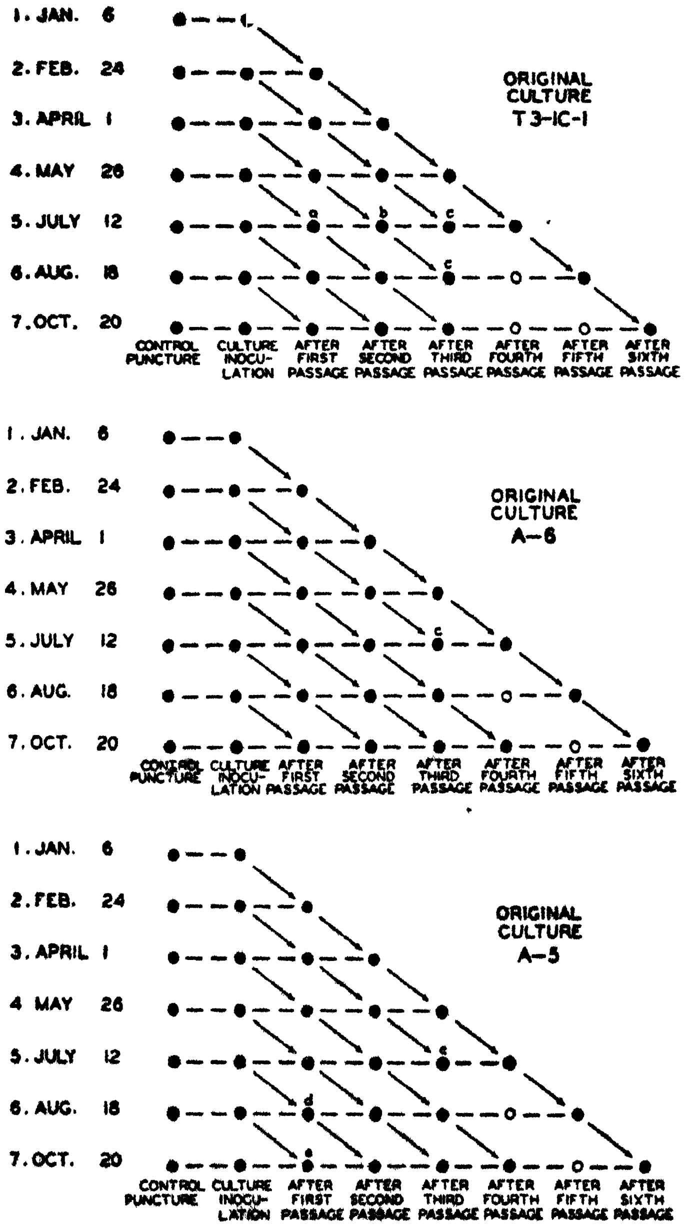

FIG. 2. PROCEDURE IN THE STUDY OF SUCCESSIVE PLANT PASSAGES WITH THREE DIFFERENT CROWN-GALL CULTURES

a. Culture T3-d2 isolated (table 1). *b*. Only one plant had a small crown gall. *c*. No crown galls were present on either plant. *d*. Culture A5-E7 isolated (table 1). *e*. Cultures A5-F1 to A5-F7 isolated (table 1).

lated with macerated gall tissues. Isolations were made from the punctures in these plants, but only contaminating forms were present. It appears that no galls were formed because of an insufficient volume of the right bacteria to induce gall formation.

The pathogenicity and certain physiological characters of the organisms isolated from the several plant passages are described in the following pages.

Five differential media were selected for the study of their physiological behavior: (1) Ferric-ammonium-citrate potassium-permanganate broth, (2) litmus milk, (3) potassium-nitrate glycerol broth plus phenol red, (4) calcium glycerol phosphate agar, and (5) yeast-water mannitol aniline-blue agar.

The pathogenicity of the 313 cultures isolated from plant passage was determined on young tomato plants by the same method previously described. Of these cultures, 70 were isolated from punctures that had not formed galls and which were not typical crown-gall cultures in appearance nor in physiological and pathogenic behavior. They were considered as contaminating organisms and were discarded. Two hundred and thirty-four cultures produced typical crown-gall reactions in the physiological media and in pathogenicity tests. The nine remaining cultures showed physiological variations but produced typical galls. In every case, these cultures were isolated after the first plant passage. Since these cultures showed physiological variations, single-cell cultures were isolated from several of them.

Certain physiological characters and the pathogenic behavior of these nine aberrant cultures, and of the single-cell progeny of certain of them, are compared with typical crown-gall and radiobacter reactions in table 1. From this table it is apparent that these cultures vary from the normal in one or more of the following characteristics: failure to produce serum zone in milk, failure to absorb aniline blue, the production of an alkaline reaction and consequent pink color in the potassium-nitrate glycerol phenol-red medium and "rough" colonial character. In all other physiological characteristics observed and in pathogenicity these cultures were similar to typical crown-gall cultures. None of the aberrant cultures showed more than two characters at variance from the normal.

None of the cultures which showed typical crown-gall physiological reactions were avirulent, indicating that continued plant passage did not reduce the pathogenicity of the organism.

TABLE 1

Summary of variations in several bacteriological characters shown by single-cell parent cultures, the nine aberrant cultures found after plant passages, and certain single-cell progenies

BACTERIAL CULTURES EMPLOYED	BACTERIOLOGICAL CHARACTERS				
	Serum zone formed in milk	Pink in phenol-red broth	Aniline blue absorbed	Rough agar colonies	Crown-gall produced on tomato
Original single-cell culture T3-1c-1	+	−	+	−	+
(1) After one plant passage T3-d2*	+	−	+	+	+
Four single-cell progenies	−	−	+	+	+
Three single-cell progenies	+	−	+	+	+
Original single-cell culture A5	+	−	+	−	+
(2) After one plant passage A5-E7†	+	+	−	−	+
Eleven single-cell progenies	+	+	−	−	+
One single-cell progeny	+	−	−	+	+
(3) After one plant passage A5-F1‡	+	+	−	−	+
(4) After one plant passage A5-F2‡	+	+	−	−	+
(5) After one plant passage A5-F3‡	+	+	−	−	+
(6) After one plant passage A5-F4‡	+	+	−	−	+
(7) After one plant passage A5-F5‡	+	+	−	−	+
Two single-cell progenies	+	+	−	−	+
One single-cell progeny	+	−	−	−	+
(8) After one plant passage A5-F6‡	+	−	−	−	+
(9) After one plant passage A5-F7‡	+	+	−	−	+
Bacillus radiobacter R3-1	+	+	−	−	−

* Culture isolated after first plant passage (*a*, fig. 1) of culture T3-1c-1.
† Culture isolated after first plant passage (*d*, fig. 1) of culture A5.
‡ Culture isolated after first plant passage (*e*, fig. 1) of culture A5.

Both rough and smooth pathogenic cultures were obtained from isolations. Rough cultures which are similar to the normal pathogenic smooth cultures except for roughness and others which are rough and are similar in all physiological reactions except the production of the clear serum zone in litmus milk were isolated.

Smooth, pathogenic crown-gall cultures which are similar to *B. radiobacter* physiologically on potassium nitrate glycerol phenol-red broth and yeast-water mannitol aniline-blue media were also obtained.

The occurrence of these pathogenic crown-gall cultures which possess physiological characteristics more closely allied to *B. radiobacter* than to the typical cultures of *P. tumefaciens* is another indication of the close relationship between the crown-gall and radiobacter organisms. Earlier in this paper a culture was described which was a typical crown-gall bacterium in its physiological characters but resembled *B. radiobacter* in being non-pathogenic.

THE RELATIONSHIP OF PHYTOMONAS TUMEFACIENS, P. RHIZOGENES AND BACILLUS RADIOBACTER

Superficial examination of cultures of the crown-gall, hairy-root and radiobacter organisms might indicate that these organisms were identical. The new physiological tests reported here, together with others reported earlier and with the plant pathogenicity tests, show that these organisms are not identical either in their physiological characters or in their pathogenicity.

Pathogenicity reactions would indicate that the crown-gall and hairy-root organisms are closely related, since both stimulate growth. Radiobacter does not attack any of the plants tested and in this respect is quite different from the other two organisms. A classification on the basis of physiological reactions would place the radiobacter and crown-gall organisms very close to each other, with the hairy-root organisms less closely related.

Further evidence of the close relationship of these three organisms is given by a study of the atypical cultures which have been described in this paper. In the original group of cultures T2-1b and its single-cell progeny all reacted as crown-gall bacteria in the physiological tests but failed to infect any of the plants tested. A similar culture was secured by picking a colony from a poured plate of a single-cell typical crown-gall culture. These cultures have the physiological characters of crown-gall bacteria but exhibit the lack of pathogenicity which is characteristic of served involved (1) smooth and rough characteristics, (2) reaction in potassium nitrate glycerol phenol-red broth, (3) dye absorption in yeast-water mannitol aniline-blue medium, and (4) the formation of a serum zone in milk.

Plant passage had no apparent effect upon virulence.

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BACTERIAL VARIATION WITH SPECIAL REFERENCE TO BEHAVIOR OF SOME MUTABILE STRAINS OF COLON BACTERIA IN SYNTHETIC MEDIA

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The name *B. coli mutabile* was employed by Neisser (1906) to designate a non-lactose-fermenting coliform organism which suddenly acquired the capacity to ferment lactose. The mutation-like variation became manifest through the formation of secondary colonies on lactose agar. Subcultures from a secondary colony proved to be active fermenters while similar cultures from the remainder of the colony had no such capacity. Other workers have since shown that this mode of variation occurs in a wide variety of bacterial species and that various carbon compounds may be involved.

Since the term "mutabile" denotes variation by mutation, Burri (1910) objected to it and proposed the name, "imperfectum," for a form which varied to sucrose in a similar manner. While Burri's contention that the term is a misnomer may be well founded, it has been so widely used that it will doubtless survive.

Our knowledge of bacterial variation has been extended through the study of various mutabile strains but there are still differences of opinion which are difficult, if not impossible, to harmonize. Previous workers have based their conclusions on experiments conducted with complex media containing peptone as well as the carbon compound to which variation occurs. Hence, it seems that little or nothing is known concerning the ability of such organisms to attack specific compounds when supplied as the sole source of carbon.

Much has been written on the problem of whether the specific sugar or alcohol is attacked from the beginning of a culture or only after a period of contact with it but no very definite experimental evidence is available and opinions differ. Similarly, it is not known whether the carbon compounds serve as a definite, specific inciting stimulus to variation or merely as a selective agent. The factors involved in formation of secondary colonies are more difficult to analyze on complex than on simple synthetic media.

The present report is based on a study of eleven strains of colon bacteria variable to lactose or sucrose, two strains of *B. paratyphi B* variable to raffinose, and three strains of *B. typhi* variable to rhamnose.

The colon strains were isolated from various sources including soil, water, feces, and a sample of cheese suspected of having been the cause of some mild cases of food poisoning. The lactose-positive strains of colon bacteria were isolated by plating on eosin-methylene-blue agar, with or without preliminary enrichment in lactose broth. Colonies were selected for isolation which either produced no color or lacked the typical metallic sheen. After replating from dilute aqueous suspensions, stock cultures were isolated and maintained on beef-extract peptone agar. All strains chosen for subsequent experiments caused delayed fermentation of lactose and formed secondary colonies on lactose peptone agar (Fig. 6). The sucrose-variable strains were discovered among some stock cultures isolated from human feces. With three exceptions the several strains had been in culture for about two years before opportunity was afforded to perform the experiments.

Before beginning the experiments proper, such differential tests were made as are necessary for classification according to the simplified system of Levine (1921). The characteristics are shown in table 1. The collection is seen to be heterogeneous. A similar condition has been reported by Kriebel (1934) for a long list of non-lactose-fermenting bacteria. It is probable that any species of either the coli or aerobacter sections may occur in the mutabile form.

Ability to utilize the homologous carbon compound was tested in a synthetic medium containing ammonia as a source of nitrogen.

The mineral base, prepared according to the formula of Frazier and Rupp (1928), and the sugars were separately sterilized. Tests were made in the liquid synthetic medium and with the addition of 2.0 per cent of bacto agar. Inoculums were prepared from young agar slant cultures by suspending in sterile water in such concentration that the inoculated liquid medium contained about one hundred cells per cubic centimeter and plates not more than two hundred cells per plate.

TABLE 1

Reactions of mutabile strains of colon bacteria

	GLUCOSE	LACTOSE	SUCROSE	SALICIN	GELATIN	MOTILITY	V.-P.	METHYL RED	SOURCE
1. *Aerobacter cloacae-mutabile*	+	−+	+	+	+	+	+	−	Water
2. *Aerobacter cloacae-mutabile*	+	−+	+	+	+	+	+	−	Water
3. *Aerobacter cloacae-mutabile*	+	−+	+	+	+	+	+	−	Soil
4. *Escherichia acidi-lactici-mutabile*	+	−+	−	−	−	+	−	+	Water
5. *Escherichia acidi-lactici-mutabile*	+	−+	−	−	−	+	−	−	Feces
6. *Aerobacter cloacae-mutabile*	+	−+	+	+	+	+	+	−	Water
7. *Escherichia coli-mutabile*	+	−+	−	+	−	+	−	+	Feces
8. *Escherichia coli-mutabile*	+	−+	−	+	−	−	−	+	Cheese
9. *Escherichia coli-mutabile*	+	−+	−	+	−	−	−	+	Cheese
10. *Escherichia communior-mutabile*	+	+	−+	+	−	+	−	+	Feces
11. *Escherichia coscoroba-mutabile*	+	+	−+	−	−	−	−	+	Feces

+ denotes prompt fermentation with acid and gas; −+, delayed fermentation with acid and gas; −, no fermentation.

All strains except no. 7 proved to be capable of growth in synthetic media, in the first as well as successive serial transplants. The number of colonies in synthetic agar plates was equal to the number in control plates containing beef-extract peptone agar. Colonies of aerobacter strains developed an acid reaction to brom-thymol-blue within forty-eight hours. Sucrose-variable strains of the coli section were somewhat more vigorous than strains variable to lactose and formed acid. Strains 4, 5, 8 and 9 produced very small streptococcus-like colonies almost invisible to the unaided eye even when streaked on the surface. Reaction of the agar remained alkaline, and colonies were bluish white. Within a period of five to eight days, secondary colonies began to appear. These remained small, little more than minute papillae (fig. 4), except in those strains which formed small primary colonies. In this case secondary colonies soon overgrew and completely obscured the primary growth, at the same time causing an acid reaction.

Growth in liquid synthetic media was found to depend on suitable aeration. When light inoculations were made into deep layers of liquid in culture tubes or flasks, irregular results were often obtained. It sometimes happened that initiation of growth was greatly delayed or completely failed. However, growth was always obtained in shallow layers even when few cells were introduced.

TABLE 2

Growth rate of Aerobacter cloacae-mutabile

HOURS	LACTOSE SYNTHETIC AGAR	PEPTONE 1 PER CENT	PEPTONE 1 PER CENT PLUS LACTOSE 1 PER CENT
0	122	80	74
12	4,700	210,000,000	225,000,000
24	26,000,000	870,000,000	1,050,000,000
36	87,000,000	1,340,000,000	2,060,000,000
48	800,000,000	1,200,000,000	4,600,000,000
60	920,000,000	1,100,000,000	5,500,000,000
72	800,000,000	1,050,000,000	5,800,000,000
84	740,000,000	920,000,000	5,600,000,000
96	660,000,000	850,000,000	2,840,000,000
120	100,000,000	620,000,000	530,000,000

The growth rates of strain number 1 in lactose synthetic medium, peptone water 1 per cent and peptone 1 per cent plus lactose are shown in table 2. There was no great difference between peptone water alone and peptone plus lactose during the first 24 hours but following this, a much more rapid rate developed and a higher maximum number of cells was produced. Likewise the death rate occurred more rapidly due to the high acidity developed after the third day. Growth in the synthetic medium was slow during the first 24-hour period, but eventually reached a maximum which was not markedly different from that in peptone water. The reaction became acid after about 84 hours accompanied by rapid depopulation.

Plating from the lactose tubes to eosin-methylene-blue agar gave lactose-positive variants which were easily recognized by greater size as well as characteristic color. Variant races produced the full acid reaction in lactose peptone water within 12 hours. Sub-races were isolated from each of the several strains by plating on the appropriate sugar medium from tubes or suspensions of secondary colonies. In all cases the variants grew more vigorously than the originals on media containing the appropriate sugar and caused prompt fermentation. In most cases the lactose-positive strains failed to form the typical metallic sheen on eosin-methylene-blue agar. Most frequently the colonies developed a deep purple center surrounded by a more or less colorless border. In some cases minute papillae formed on old colonies of this type, from which typical colonies were obtained by plating. It was also noted that some secondary colonies developed the so-called tertiary colonies with age. It appears that a full capacity to attack lactose is acquired in such cases by a series of stages. Variant colonies of all aerobacter strains were typical of the normal for this species.

These experiments show that mutabile strains of colon bacteria are able, in most cases, to utilize the sugar to which variation occurs and in some cases to form acid. The latter point is difficult to determine for the reason that small amounts of acid may be completely neutralized by alkali; likewise because variation soon causes a mixture of variant and original cells. The single strain which made no growth in lactose synthetic media will be discussed more fully in a subsequent section.

THE RÔLE OF SPECIFIC CARBON COMPOUNDS IN VARIATION

Since mutabile strains of bacteria, when cultivated in media containing the appropriate sugar or alcohol, yield an abundance of positive variants, the conclusion has generally been reached that a carbon compound to which an organism is variable exerts some sort of a specific, inciting stimulus to variation. There is little definite proof that this is true, but we have generally accepted it in the absence of a better explanation.

Burri (1910) explained such variation as due to the activation of a latent ability to secrete a specific enzyme through stimulation derived from the sugar. The capacity once activated for any cell is transmitted to its progeny. Stewart (1927) concluded that lactose or other sugar is wholly without influence except at a certain brief period in the life of a culture when its vegetative activity ceases and a process of segregation and autogamic conjugation occurs. The sugar if present at this time influences the segregation of characters. The experimental evidence presented in support of this hypothesis is not convincing. Reiner Müller (1911) invoked the side-chain theory to explain the specific effect of rhamnose on the variation of *B. typhi.*

An examination of the meager evidence in support of a specific inciting stimulus reveals the fact that we are dealing with an hypothesis rather than an established fact. Direct experimental proof of the truth of this hypothesis is difficult, if not impossible to obtain, but if it can be shown that such variation occurs in the absence of the specific sugar, then the theory of a definite inciting stimulus will have lost its chief support.

It has been suggested by some workers, Smith (1913) and others, that variation occurs spontaneously in the absence of any specific sugar, but if the sugar is present it acts as a selective agent, since the variant cells are benefited by it, and are thus enabled to multiply more rapidly than the original type. This might result in slow but steady transformation of the population until variant cells would eventually predominate. This explanation has much to recommend it on theoretical grounds, but has met no very general favor because of inadequate experimental proof. Nungester and Anderson (1931) studied a coliform bacillus which varied with respect to lactose, and obtained lactose-positive variants in one instance by plating to lactose agar from a sucrose broth culture. Havens and Irwin (1932) were able to isolate both sucrose-positive and negative strains from plain agar cultures of *Salmonella Morgani.* They concluded that variation occurred spontaneously under ordinary conditions of cultivation with no apparent environmental stimulus. Burri (1909) studied a culture of Neisser's original *B. coli-mutabile* and found both lactose-positive and negative colonies when plain agar cultures were plated on Endo's agar. He concluded that the culture consisted of a mixture of two types of cells. On the other hand, he believed that all cells of his *B. imperfectum* were alike in non-fermentation of sucrose and could acquire this capacity only through the stimulus of sucrose.

It has been mentioned above that one of our mutabile strains proved to be incapable of growth in lactose synthetic media when inoculated with a small number of cells. This strain caused fermentation more promptly than others, within 36 hours, when cultivated in lactose peptone water, and formed secondary colonies on lactose agar on the second day. An experiment was performed to determine whether cultures grown on plain agar contained any cells, of variant or original type, capable of utilizing lactose as a sole source of carbon. This experiment was based on the method of Braun and Cahn-Bronner (1922) who succeeded in obtaining ammonia-assimilating strains of *B. paratyphi A* and *B. typhi* by heavy inoculations and serial transplants in a medium containing ammonia as the only source of nitrogen. Neither organism was capable of growth in this medium when inoculated in the usual way, but when enormous numbers were introduced, some few cells were able to grow. Pure cultures of ammonia-assimilating strains could then be obtained.

The present experiment is a modification of this method. A 24-hour agar slant culture was washed down with 10 cc. of water and further diluted by steps of ten to include the complete range from one tenth to one billionth part of the culture. All dilutions were plated in glucose as well as lactose synthetic agar in order to determine with certainty that failure to obtain growth in the preceding experiment could not have been due to the nitrogen source. The highest dilutions were plated in peptone beef-extract agar to determine the number of cells introduced into the plates of lower dilutions. Computing from the plain agar series, it was estimated that the lowest dilution contained about three billion cells per cubic centimeter. Growth occurred throughout the range in glucose synthetic agar. The colonies in plain agar and glucose synthetic agar were practically equal in the high dilutions. Lactose synthetic agar supported growth in low dilutions only. Plates above the 100,000 dilution were sterile. The number of colonies in plates which supported growth was proportional to the dilutions. It was determined that not more than one cell per 100,000 could grow in this medium.

The colonies grew vigorously with formation of acid. Transplants to eosin-methylene-blue agar gave typical metallic colonies in some instances, while in others the colonies were purple or with purple centers. Shake cultures in lactose peptone agar produced acid and gas within 12 hours. It was thus shown that cells of two types were present in the original culture. All cells capable of growth yielded races which could not be distinguished from variants isolated by plating from secondary colonies.

There seemed no reason to believe that the culture consisted of a mixture of two unrelated strains. However this possibility had to be excluded before we could conclude that variation occurs spontaneously without the influence of lactose.

Two methods of experimentation were available, namely, isolation of pure strains by single-cell methods or testing numerous colonies from ordinary plain agar plates. Since the latter appeared likely to afford reliable results, the experiments were carried out by this method. The previous experiment had shown that variants are present in small numbers only. It seemed, therefore, that plating to plain agar from high dilutions would insure separation of the two types. Accordingly, plate cultures were prepared from a 24-hour broth culture diluted one to one hundred million. Such plates contained about 8 to 10 colonies, not more than 2 or 3 of which grew on the surface. Each colony to be tested for variants was scraped off as completely as possible and emulsified in 100 cc. of sterile water. The number of cells capable of growth in lactose synthetic agar was determined by plating 1 cc. of the suspension in this medium and the total number by plating appropriate higher dilutions in beef extract peptone agar and glucose synthetic agar. The results are shown in table 3.

It is seen that all colonies contained variants and that the proportion of original to variant cells was relatively uniform.

In addition to this experiment, numerous colonies from plain agar plates were tested by taking up as much of the growth as possible, suspending it in 10 cc. of water, and plating 1 cc. in lactose synthetic agar. This series included 100 colonies from plain agar plates prepared from high dilutions as described above. All colonies contained variant cells in about the same number per colony shown in table 3. Thus it is proved that variation occurs in this strain in the absence of lactose. The variant cells, however, are so few in number as to escape detection by conventional methods of plating.

TABLE 3

Number of variant and original type cells in plain agar colonies of B. coli-mutabile as determined by capacity for growth in lactose synthetic, glucose synthetic and beef extract peptone agar

COLONY	KIND OF MEDIUM AND NUMBER OF CELLS PER COLONY CAPABLE OF GROWTH		
	Lactose synthetic agar	Beef extract agar	Glucose synthetic agar
1	6,300	3,540,000,000	3,420,000,000
2	3,300	1,610,000,000	1,640,000,000
3	2,900	730,000,000	710,000,000
4	2,200	840,000,000	860,000,000
5	2,080	1,200,000,000	1,100,000,000

In order to determine whether variation could occur in a medium without either peptone or lactose, a culture was carried in glucose synthetic liquid medium with daily transplants for a period of 10 days. At the end of this period the last tube of the series was plated in glucose synthetic agar. After an incubation period of 48 hours, single colonies were suspended in sterile water and plated in lactose synthetic agar. The number of variant cells was approximately the same as in colonies from beef-extract peptone agar. It appears that variation occurs under any conditions which are suitable for growth.

The method of plating from low dilutions was applied to the remaining strains which had been found capable of growth in lactose synthetic media. In this case it was necessary to distinguish variant colonies from the original type since both could make some growth even in such densely seeded plates. Colonies of the original type were invariably small, requiring microscopic examination to reveal their presence while variant colonies could be seen with the unaided eye or by means of low magnifications with a wide field binocular. The relative sizes in a typical plate are shown in figure 3. In addition to differences in size the variant colonies became much more deeply yellow than the originals which remained pale yellow or greenish colored. The number of variant colonies could be determined with almost the same accuracy as in the case where no colonies of the original type developed. Some differences were found in the number of variants but single well separated colonies of all strains on plain agar contained such cells in a proportion not greater in any case than about one variant per hundred thousand original cells.

In some cases it was noted that deep variant colonies became surrounded by a definite halo caused by stimulating the growth of colonies of the original type. (Fig. 5.) The halo zone could not be observed until the colonies reached an age of about 48 hours, and required several days to arrive at the maximum diameter, which might be as much as one or more centimeters. Such a complex might appear to the unaided eye as a single large colony. This phenomenon seems to depend on hydrolysis of the sugar which makes it more readily available for the surrounding colonies. It probably signifies the most fundamental difference between original and variant type cells.

The method thus far described of testing a colony for variants is limited to organisms which are capable of utilizing ammonia as a source of nitrogen. Since some mutabile strains of bacteria require a more complex source of nitrogen, a test was made to determine whether or not variants could be distinguished in peptone media containing sugar. As in the cases already reported, heavy seeding was employed. Media containing 1.0, 0.1 and 0.01 per cent peptone were tested. With two exceptions the differentiation was almost as satisfactory as in synthetic media. Strains 1 and 10 showed little or no difference in size of colonies. There was no special advantage noted in the smaller amounts of peptone for any of the positive strains. It appeared that somewhat more colonies developed in the medium containing greater amounts of peptone.

It is known that *B. paratyphi B* utilizes ammonia and also varies with respect to raffinose in the same manner as mutabile strains of colon bacteria vary to lactose and sucrose. Two strains only were available for testing. The history of these strains is not well known but they have been carried as stock cultures in this laboratory for many years. When plated out on beef-extract peptone agar containing 0.5 per cent of raffinose, one of the strains produced two types of colonies, about equally numerous, which differed in vigor of growth. The more vigorous colony produced no secondary colonies, while the relatively thinner and less vigorous type produced them in great abundance. The second stock culture showed but one type when plates were streaked to give well scattered colonies. Secondary colonies developed abundantly on all primary colonies. This strain was used for further tests. It grew well in raffinose synthetic media, producing as many colonies in the synthetic agar as in beef-extract peptone agar inoculated with an equal number of cells. It is shown, therefore, that *B. paratyphi B* resembles the majority of mutabile colon strains in its capacity to utilize the carbon compound with respect to which variation occurs.

It remained to be determined whether variation occurs in a medium without raffinose. Although it was anticipated that synthetic raffinose agar would be found preferable to media containing peptone, such was not the case. In heavily seeded plates the colonies were all minute and variants were not readily distinguishable. When the medium contained 1.0 per cent of peptone no very clearly marked difference in size could be noted. A medium containing 0.1 per cent of peptone and 0.5 per cent of raffinose was found to give a sharp differentiation. This medium was employed to test colonies which had produced secondaries on raffinose agar as well as whole colonies from plain agar. Variant cells in the latter case rarely exceeded about 100 or 200 per colony.

B. typhi varies with respect to rhamnose but in most cases does not assimilate ammonia. Three stock cultures including the Rawlins strain were plated out on beef-extract peptone agar plus 0.5 per cent of rhamnose. Each produced numerous secondaries and the primary colonies appeared to be of uniform type. These strains were tested for variants in plates heavily seeded from single colonies which had grown on beef-extract peptone agar. A medium containing peptone 1.0 per cent plus rhamnose 0.5 per cent was found suitable. Original type colonies in such densely seeded plates were very minute while those of the variant type were easily seen with the unaided eye. Not more than 50 to 100 large colonies were obtained in plates seeded with a whole colony from plain agar.

It should be noted that in all cases the large colonies were proved to be pure variants by establishing cultures and applying the necessary differential tests. Since secondary colonies are able to develop within the mass of a primary colony it is not surprising that they should grow more vigorously than the original type in densely seeded plates. Such plates are almost if not quite analogous to a single giant colony.

The fact that variation to specific carbon compounds occurs in mutabile strains independently of the compound has not been generally recognised. Burri (1909) reported some experiments which deserve mention in this connection. His conclusions have been frequently quoted but seem to be mainly erroneous.

The experiments upon which Burri based his conclusions were carried out in tube cultures of a sucrose-variable organism of the mutabile type. When shake cultures were made in sucrose agar with heavy seeding, (about ten million cells) he observed both small and large colonies. The latter were few in number, about 30 to 100, and were not visible to the unaided eye until the 4th or 5th day. Delayed fermentation occurred simultaneously with development of the large colonies. He next prepared serial dilutions such that each successive dilution contained one tenth the number of cells in the preceding one and inoculated a series of sucrose agar shake tubes. He observed that the number of large colonies did not decrease in proportion to the dilutions and hence such colonies could not be explained on the basis of any difference in capacity to utilise sucrose at the beginning.

He reasoned that the determining factor must be environmental rather than difference in potentiality of the cells since all of the colonies became large when the number was small but only a few large colonies developed in heavily seeded tubes. His fundamental error was failure to recognize that the large colonies which developed in thickly-seeded tubes consisted of pure variant cells whereas the large colonies in the sparsely-seeded tubes, where all became large, contained both variant and original cells. He does not appear to have studied sufficiently the careful work of Neisser (1906) and Massini (1907) for there is no evidence throughout the paper that pure variant type subraces were ever established. His conclusion that the transformation is a gradual rather than a sudden mutation-like process was based on the fact that young colonies transferred to shake tubes required a longer period for fermentation than similar colonies which had become older. He appears to have believed that the colony as a whole was transformed due to the influence of sucrose on the formation of enzymes. There is no reason to believe that the phenomenon which Burri described differed in any way from that reported above. The evidence is conclusive that variation of mutabile strains to a given specific carbon compound occurs spontaneously in media from which the compound is absent. We are obliged to conclude, therefore, that sugars or alcohols act as specific selective agents rather than as stimulators of variation. It appears that an organism which has no capacity for spontaneous variation to a carbon compound is not caused to vary to it by cultivation in contact with the compound no matter how long the contact may be prolonged. Burri's conclusions that each and every cell of a culture is capable of becoming transformed into a lactose fermenter could not have been reached if he had taken the pains to isolate strains from the different components of colonies.

ENHANCEMENT OF FERMENTING POWER BY SERIAL TRANSPLANTS

We have long been accustomed to regard the method of transplanting serially to fresh media, at short intervals, as a certain means for invigorating cultures. More recently this has become a prevailing method for obtaining so-called dissociation. Various reports have been made regarding enhancement of power to cause fermentation when slow fermenters are transplanted serially in sugar media. The literature of this subject has been reviewed in a recent paper by Kriebel (1934). It appears that some strains are capable of enhancement in this manner while others are not.

Previous reports indicate that all the factors involved have not been well recognized. Earlier workers appear to have sought enhancement through invigoration of the culture as a whole without due regard to variation and the isolation of variants. Kriebel (1934) has shown that some slow lactose-fermenting strains dissociate in the same manner as mutabile strains to form true *B. coli*. There is every reason to expect that such strains would prove capable of enhancement by serial transplants under certain experimental conditions but not under others. There is little reason to expect that a strain which fails to cause fermentation in standing cultures would ever become enhanced by serial transplants.

Our aerobacter strain 1 has been studied with reference to the factors involved in enhancement by transplanting serially in lactose peptone water. This strain requires a period of 72 hours to reverse the reaction of neutral peptone lactose water containing peptone 1 per cent, lactose 1 per cent, and dipotassium phosphate 0.25 per cent with brom-thymol-blue as indicator. It has already been shown that cultures of this organism at the end of 24 hours incubation have reached a population of about one billion cells per cubic centimeter when inoculated with about 100 cells. Plating from the tube at this time reveals the fact that variant cells are so few as to escape detection by conventional methods. Plating to lactose synthetic agar from whole colonies grown on lactose peptone agar and on plain agar showed that more variants are present in the former but the numbers are not strikingly different. Even at 48 hours, original cells are greatly in the majority. It becomes obvious, therefore, that we need only dilute the culture to proper proportions in order to eliminate the variant cells. If the original type cells have not profited by their sojourn in the lactose medium, then each new tube of the series starts with no greater probability of prompt fermentation.

It was determined by experiment that dilutions of 1, 10, or 100 million could be carried forward in 1-cc. portions at 24 hour intervals for a period of 30 days without influencing the period required for fermentation. On the other hand dilutions of 100,000 shortened the period to about 36 hours while still lower dilutions caused rapid transformation.

The time factor is of less importance since transplants made at 12 hours shortened the period equally well provided the dilutions were not so high as to exclude all variant cells. Transplanting from tube to tube with the tip of a straight needle might lead to erroneous conclusions.

Whether fermentation occurs promptly or is long delayed depends on the relative numbers of fermenting and non-fermenting cells present. This depends on the rate of variation and difference in relative growth rates of the original and variant cells in a medium in which the latter have a decided advantage.

The relative number of variant and original cells necessary to cause a rapid fermenting mixture was determined for aerobacter strain 1 by mixing cells in known proportions. The original strain and one of its lactose-positive variants were cultivated separately in beef-extract peptone broth for a period of 24 hours. The mixture was then prepared which contained 9 cc. of original and 1 cc. of variant. This was diluted serially in steps of 10 to 100 million. Tubes containing 20 cc. of lactose peptone phosphate broth were inoculated with 1.0 cc. of the dilutions. This proportion caused fermentation within 12 hours regardless of the total number of cells introduced. A 5.0 per cent mixture similarly prepared required 24 hours while with only 1.0 per cent the period lengthened to 36 hours.

These results indicate that a culture of the original strain inoculated in the usual manner has reached a ratio of about 1.0 pre cent after 36 hours, 5.0 per cent in 48 hours and 10 per cent in 60 hours since the average time required is 72 hours. This number correlates roughly with determinations made by plating methods.

Serial transplanting appears to be a method of doubtful value for enhancement of fermentation, since the indications are that this does not depend on invigorating the population as a whole but on the building up of variants. This can take place as well or better in standing cultures. In any event the success of serial transplanting depends on the number of cells carried forward.

A point of special interest has been brought out in the investigation of Kriebel (1934). Fermentation occurred more promptly in a medium containing 5.0 per cent of lactose than with greater or lesser amounts. This was found to be true with the strains of colon bacteria considered here. This might seem to support the notion of a direct effect of lactose on the cells as a specific inciting agent. It is as logical, however, to regard the effect as selective.

SECONDARY COLONIES

In a previous report (1933) I have set forth the factors involved in the formation of secondary colonies. At that time it was not definitely known whether this phenomenon could occur on synthetic agar. That such colonies may be formed in this manner, and in some cases reach a size much greater than the original, has now been shown.

Since the conditions are simplified it seems possible to analyze the factors more definitely. The primary colony remains small because the cells possess a limited capacity for utilization of lactose. The same is to be seen when any bacterial species is placed in an unfavorable nutritive medium. As the medium becomes depleted, and an unfavorable reaction develops, growth of the colony ceases. During its limited growth period, variation occurs. The number of variant cells produced must be very small based on the law of probability. These cells, because of a greater capacity for utilization of lactose, are able to continue growth which, due to confluence, results in the formation of a single large secondary colony. Strains of *Aerobacter cloacae*, *B. coli-communior*, and *B. coscoroba*, produce rather large primary colonies and form acid. Secondary colonies remain small because the degree of enhancement is less and the acid reaction is not favorable to continued growth. Thus we arrive again at the same conclusion as formerly, that the chief factors involved in the formation of secondary colonies are depletion of nutrients and beneficial variation. We are obliged to abandon the theory of a specific inciting stimulus to variation in mutabile strains of bacteria.

NON-CORRELATED VARIATION

It is well known that dissociation of a bacterial species in which colony form is involved may be accompanied by changes in various other characteristics. Hadley (1927) has given a full review of this subject. On the other hand, there are cases known in which such correlation could not be shown. Nungester (1933) gave a list of such cases which he had investigated. There seem to be few observations on mutabile strains with regard to such behavior.

All aerobacter strains included in this study were found capable of spontaneous variation from smooth to rough and back to smooth with intermediate stages. No special study was made of the conditions which might influence the change from the normal smooth to the rough type. A few daily transplants in beef-extract peptone broth sufficed to cause reversion from rough to smooth colonies. Rough colonies were frequently obtained by plating broth or agar cultures.

Two types of rough colonies were distinguished (figs. 1 and 2). The more common type formed tough membraneous colonies which could be lifted bodily from the agar by means of a needle. Such colonies could not be broken up to form a uniform suspension in salt solution. The colonies were further characterized by definite concentric rings of growth culminating in a crater-like depression at the apex, surrounded by a heavy crown (fig. 1).

The rough characteristics were slightly more pronounced on agar containing lactose but secondary colonies developed as in the smooth parental type and fermentation in liquid media was *no more rapid than before. Subculturing from* secondary colonies or liquid cultures yielded a rough race capable of rapid fermentation.

A similar smooth membraneous form which lacked the concentric rings of growth and had the form of a hollow dome was frequently observed. Although the colonies were smooth the membraneous character seems to indicate an intermediate form between the typical smooth and rough condition. A second rough type, also in the form of a membrane (fig. 2), developed frequently on plain or lactose agar plates. In this case the membrane was thin and flat or slightly raised at the center. There were no concentric rings but from 8 to 10 plates placed at right angle to the surface radiated from center to circumference. These colonies formed secondary colonies when cultivated on lactose agar and produced a lactose-positive race of similar form.

In addition to the rough colonies a smooth lactose variant could always be obtained by plating from old colonies growing on lactose agar or from lactose peptone broth. Colonies of this type were large, almost transparent, and of a watery consistency on plain lactose agar. On eosin-methylene-blue agar, the colonies were easily distinguished by size, consistency and color. The several colony types described above together with the original might be observed on a single plate prepared from a liquid medium containing lactose. There was thus presented a confusing array of types similar to that which has been reported by Nungester and Alexander (1931) for a coliform species in which they described many different colony types.

GENERAL

The subject of bacterial variation and heredity has reached an almost hopeless state of confusion. Almost every possible view has been set forth and there seems no reason to hope that any uniform consensus of opinion may be reached in the immediate future. There are many advocates of a Lamarckian mode of bacterial inheritance while others hold to the view that it is essentially Darwinian. The early workers regarded variation in so-called mutabile strains as mutation in the sense of DeVries. Some more recent workers have explained this behavior as due to Mendelian segregation, while others have regarded it as evidence of a cyclogenic life history.

The experiments reported above support the view that variation occurs spontaneously, in some cases at least, and without regard to environmental influences. Variation, when beneficial, may be preserved by selective action of the medium. Non-beneficial variation, such as poor adaptation to nutrients, would be eliminated by overgrowth of the more vigorous original cells.

SUMMARY

Eleven mutabile strains of colon bacteria, two strains of *B. paratyphi B*, and three strains of *B. typhi* have been studied. All strains of colon bacteria and *B. paratyphi B* grew in synthetic culture media containing ammonia and the carbon compound to which variation occurs. One strain of *B. coli-mutabile* consisted of a mixture of cells, the majority of which could not utilize lactose.

Sugars and alcohols act as selective agents but do not exert a specific inciting stimulus to variation. Variant cells were invariably present in plain agar colonies but in such small numbers as to escape detection by conventional methods. The number of variant cells in a colony can be determined by means of densely seeded agar of appropriate formula.

Secondary colonies are formed by colonies growing on synthetic agar containing the appropriate carbon compound. The factors involved in their formation are depletion of food and beneficial variation.

Enhancement of fermentation by serial transplants depends on conditions which permit building up of variant cells. The most important factor is the number of cells transplanted from tube to tube.

Variation in form from smooth to rough and back to smooth, in aerobacter strains, is not correlated with variation to lactose.

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PLATE 1

Fig. 1. Rough colony of *Aerobacter cloaceae-mutabile* at end of six days.

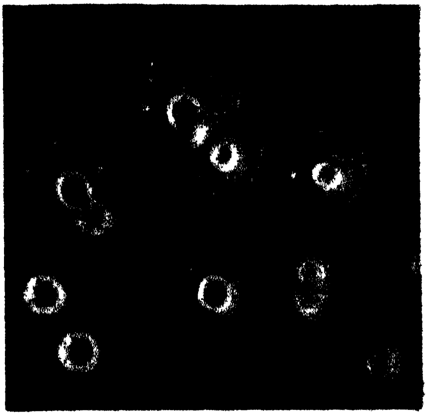
Fig. 2. Rough colony of *Aerobacter cloaceae-mutabile* at end of three days.

Fig. 3. Mixture of variant and original colonies, *Aerobacter cloaceae-mutabile*. Lactose synthetic agar heavily seeded from a lactose synthetic tube.

Fig. 4. Colonies of *B. communior-mutabile*. Colonies with minute papillae. Sucrose synthetic agar at end of eight days.

Fig. 5. Same strain as figure 4. Large sucrose-positive variant deep colony with halo of original colonies. Sucrose synthetic agar seeded heavily from a plain agar colony. Photographed at 48 hours.

Fig. 6. Colony of *B. coli-mutabile* on lactose peptone agar at end of ten days.



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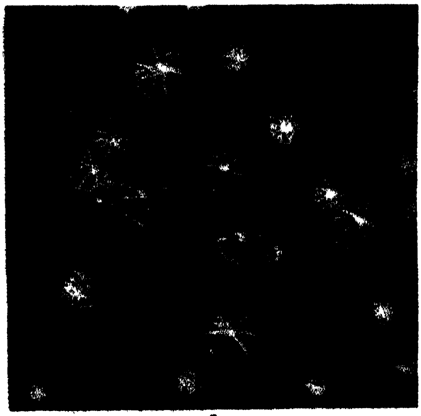

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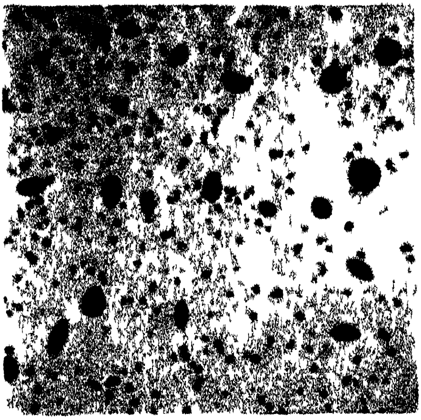

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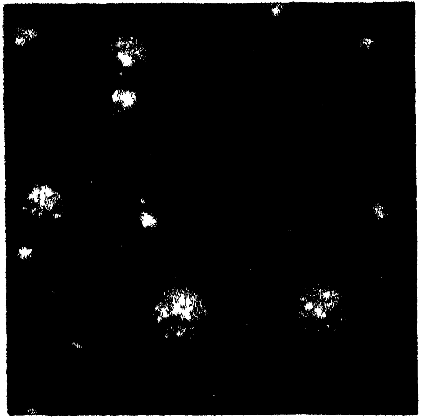

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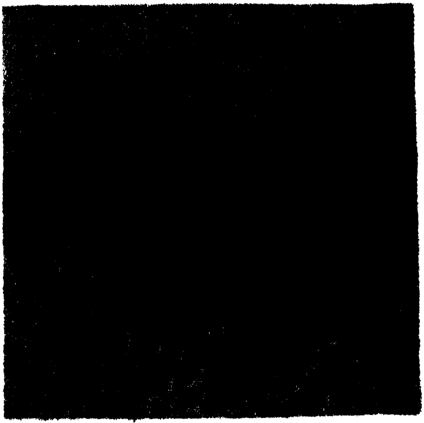

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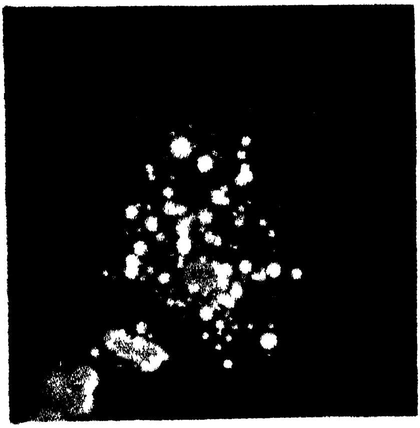

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